Structural and Functional Analysis of Zona Pellucida.

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Contents

Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree nor is any part of it being submitted concurrently in candidature for another degree. All experiments were performed at the Medical Research Council Reproductive Biology Unit in Edinburgh.

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Abstract

All mammalian oocytes are surrounded by an acellular, transparent, glycoprotein shell known as the zona pellucida. This structure plays many important roles during fertilization and embryogenesis including the mediation of sperm-egg recognition, induction of the acrosome reaction, development of the block to polyspermy and the physical protection of the oocyte/zygote throughout the preimplantation stage of development.

The ability of the zona pellucida to mediate the species specific recognition of spermatozoa is due to a glycoprotein constituent of this structure, known as ZP3. In view of its important role in the initiation of fertilization, ZP3 has been identified as a suitable candidate for the development of a contraceptive vaccine. In order to determine the validity of the marmoset as an animal model for engineering such a vaccine, it was important to establish the primary amino acid structure of marmoset ZP3 and relate this sequence to the human homologue. The strategy employed to achieve this aim involved the use of the polymerase chain reaction, marmoset ovarian cDNA, and primers derived from the human ZP3 sequence. The marmoset ZP3 gene was found to possess an open reading frame of 1272 nucleotides encoding for a polypeptide core of 424 amino acids. Marmoset (mar) ZP3 was thereby found to be exactly the same length as human and mouse ZP3 and 2 amino acids longer than the hamster homologue. The primary amino acid sequence of marZP3 was found to exhibit a high degree of homology (91%) with the human ZP3 sequence and more than 60% homology with the hamster and mouse sequences. An additional conserved feature of the ZP3 molecule was found to be the position of the cysteine residues; the human marmoset, mouse and hamster all possessing 15 cysteine residues at identical sites.

In situ hybridization studies indicated that marZP3 was expressed exclusively in growing oocytes, the ZP3 signal being much greater in secondary and Graafian follicles than in the primary follicle population and poorly expressed, if at all, by primordial follicles.

In order to generate recombinant marmoset ZP3 for immunization purposes a full length 1.3 kb insert encoding for marmoset ZP3 was cloned downstream of the malE gene to generate a fusion protein containing marZP3 and a maltose binding domain. This protein was found to be generated in a soluble form in aqueous solutions and the antigenic integrity of this molecule was demonstrated by its capacity to be recognized by a monoclonal antibody raised against human ZP3. This full length fusion protein has been purified by anion exchange chromatography and used to generate polyclonal antibodies.

In addition to these *in vitro* studies on the generation of a recombinant marZP3 for active immunization purposes, *in vivo* studies have also been performed on the induction of active immunity against porcine ZP3. These studies have demonstrated that an important side effect of induction of immunity against a heterologous ZP3 antigen involves the depletion of the primordial follicle pool. Moreover this pathology could be transferred to non-immunized recipients by the passive transfer of antibody. These data raise the important question as to whether a similar pathology would be induced if homologous zona antigens were used for the induction of immunity. As a result of the work described in this thesis, this question can now be addressed in the marmoset monkey using recombinant marZP3 as antigen.

Abbreviations

å	Alpha
ADCC	Antibody-dependent cell-mediated cytotoxicity
AR	Acrosome reaction
bp	Basepairs
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
DEAE	Diethylaminoethyl
DGZP-32	Deglycosylated 32,000 Da porcine zona pellucida
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate(s)
DTT	Dithiothreitol
ECl	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunoabsorbant assay
EßGD	Endo-ß-galactosidase-digested ZP
EtBr	Ethidium bromide
FCA	Freund's complete adjuvant
FSH	Follicle stimulating hormone
GCs	Granulosa cells
hCG	Human chorionic gonadotrophin
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase

hZP3	Hamster ZP3
huZP3	Human ZP3
Ig	Immunoglobulin
IPTG	Isopropyl-1-thio-ß-D-galactoside
kDa	KiloDalton
LiCl	Lithium chloride
LiDS	Lithium dodecylsulphate
m	Metre
mar ZP3	Marmoset ZP3
MBP	Maltose binding protein
mg	Milligram
ml	Millilitre
mM	Millimolar
mm	Millimetre
Mr =	Molecular weight
mRNA	Messenger RNA
mZP3	Mouse ZP3
nm	Nanometre
nt	Nucleotide(s)
OSP-1	Oocyte specific binding Protein-1
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethyl sulphonyl fluoride
PPZA	Purified porcine zona pellucida antigen
rec55	Rabbit recombinant ZP protein Mr=55,000

RNA	Ribonucleic acid
SDS	Sodium dodecylsulphate
SIZP	Solubilized isolated zona pellucida
ß-gal	ß-galactosidase
TAE	Tris/acetate/EDTA buffer
TBE	Tris/borate/EDTA/ buffer
TBS	Tris-buffered saline
TE	Tris/EDTA buffer
TFMS	Trifluoromethane sulphonic acid
Tris	Tris-[hydroxymethyl]aminomethane
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
XL1-B	*Escherichia coli* cells strain XL1-Blue
ZP	Zona pellucida
ZP3	A 55,000 kDa porcine Zona Pellucida protein
ZP3å	ZP3 containes two types of protein, one of them is ZP3å
ZP3å/EBGD	Endo-ß-galactosidase-digested ZP3 glycoprotein
ZP3ß	ZP3 containes two types of protein, one of them is ZP3ß
ZP3ß/DG	Deglycosylated ZP3ß
ZPå/DG	Deglycosylated ZP3å
µl	microlitre
µM	micromolar

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1 Introduction

The world's population has more than doubled in the last four decades, from 2.5 billion in 1950 to 5.3 billion in 1990, according to the latest United Nations estimates. The rate of population growth during the past 40 years, however, has not been constant. The estimated annual rate of growth increased from 1.79% in 1950 -1955 to 2.06% in 1965 -1975, but then decreased to 1.73% in 1975 - 1980. It has subsequently remained almost unchanged at that level throughout the 1980s. Whether the growth rate continues to fall or remains constant makes a considerable difference in the long run: the world population would have reached zero growth at 6.7 billion in 2030 if the growth rate decline in the 1970s had not stalled in the 1980s and continued linearly; the world population, however, will increase to 10.7 billion in 2030 if the growth rate stays on the current plateau (Horiuchi, 1992).

An important facet of the pattern of population growth is that it is largely focused on the countries of the developing world. If current trends continue it has been estimated that approximately 80% of the world's population will be living in developing countries by the year 2000. Clearly, this pattern of population increase places a considerable demand on the resources of developing countries and directly, or indirectly, has a major impact on the prevalence of environmental damage, famine, disease and poverty. In order to achieve zero population growth, new methods of contraception will have to be developed and, moreover, methods of contraception that are appropriate for the problems encountered in the Third World. In this context, a contraceptive vaccine offers many inherent advantages since it should provide prolonged protection against fertility and has the potential to provide a safe and reliable method of birth control. There are three main target areas for a possible contraceptive vaccine: (1) reproductive endocrine signals, (2) products of the conceptus and (3) gamete specific antigens. A contraceptive

vaccine based on the zona pellucida (ZP) or the mammalian sperm receptor (ZP3), a component of the ZP, offers particular promise since this target should lead to the development of a vaccine that prevents conception rather than terminate an ongoing pregnancy, as is the case with the other major approach being researched at the present time, the anti-hCG vaccine (Raghupathy & Talwar, 1992).

The ideal strategy for the evaluation of a contraceptive vaccine would be one in which an animal model, such as the marmoset monkey, was immunized with homologous ZP3 peptides that exhibited perfect sequence homology with human ZP3. In order to develop such a model the primary amino acid structure of marmoset ZP3 would have to be elucidated and compared with its human counterpart. The core of this thesis deals with this issue and presents data on the primary amino acid sequence of the marmoset ZP3 molecule. Additional parts of the thesis deal with the expression and translation of the ZP3 gene in the marmoset ovary and the consequences of inducing active and passive immunity against the ZP3 molecule, with particular emphasis on the side effects associated with this treatment in terms of the loss of primordial follicles.

In the course of this work, the PCR technique was employed to clone and sequence marmoset ZP3 (Chapter 3) using human ZP3 primers. The expression of the ZP3 gene during follicular development was then investigated using *in situ* hybridization procedures (Chapter 4). Prokaryotic expression of recombinant marmoset ZP3 (Chapter 5) and the purification and characterization of recombinant marmoset ZP3 are then described in Chapter 6. The effects of active and passive immunization with porcine ZP3 and the possible mechanisms involved in ovarian pathogenicity are finally discussed in Chapters 7 and 8.

Each individual chapter has its own introductory section with the purpose of discussing the literature directly relevant to the work described in that chapter. Thus, the general introduction provided in Chapter 2 does not set out to review the entire literature relevant to all the material contained in this thesis. It does

however attempt to provide a groundwork for accepting and appreciating the studies summarized in the rest of the thesis. Each chapter also contains a discussion of the results obtained in the context of those reported by other investigators and concludes with a short summary of the results reported in that chapter. The final chapter, Chapter 9, comprises a general discussion of the results described in the thesis in the context of work by other investigators and emphasises the areas which may be appropriate for development in the future.

2 Background

2.1 Historical Aspects

Evolution of the complex array of living creatures that inhabit the earth has depended upon the successful transfer of genetic material from one generation to another through the process of reproduction. The ancient Greeks believed that menstrual blood and semen combined to form the embryo and foetal membranes. Aristotle suggested that the semen contributed in a non-material sense. He regarded embryo development to be exclusively due to the physiology of the female reproductive tract. At the turn of the seventeenth century the English physician William Harvey wrote that all creatures come from an egg (Keynes, 1966), while in 1673 Malpighi, who worked on chick embryos, suggested that the hen's egg contained the miniature chick and that the spermatozoa stimulated this to become the mature animal (Cole, 1930). Mammalian spermatozoa were first observed by van Leeuwenhoek and Hannen (1679) the report of these authors on the "little animals of sperm" dating back to November 1677. These observations concerned fresh seminal fluid and showed that the spermatozoa swarmed as a multitude of "animalcules" propelling themselves about with serpentine movements.

De Graaf described and illustrated the ovarian vesicles in several species and fitted the ova into a reasonable scheme, which is substantially what we believe today. De Graaf used the term vesicles (vesicula) or egg (ovum) for what we now call the follicle (Setchell, 1974). In 1875 van Beneden observed the male pronucleus inside an egg cell and correctly concluded that the chromosomes of the first cleavage were

derived equally from spermatozoa and egg (Gwatkin, 1977). The arrival of phase contrast microscopy helped investigators to observe living eggs (Austin, 1951). Initially the major constraint on studying the process of fertilization was the lack of *in vitro* culture techniques, however, the subsequent introduction of such procedures made it possible to dissect the fertilization process in some detail. Dauzier *et al.* (1954) pioneered this area by producing the first rabbits derived from *in vitro* fertilized ova (Dauzier *et al.*1954). During the past two decades significant improvements in our knowledge of sperm-egg interactions have been made, which have helped promote further developments in the fields of human *in vitro* fertilization, animal breeding, spermatozoa and embryo preservation, transgenic animals and immunocontraception (Wassarman, 1988a; Henderson *et al.*1988; Kinloch *et al.*1992).

In the human the act of coitus is associated with the deposition of around 300 (human) million spermatozoa near the cervix. After ejaculation, the spermatozoa have to pass through cervical mucus, which is mostly composed of macromolecular glycoprotein filaments and, in the process, many abnormal spermatozoa are excluded from the female reproductive tract (Hanson & Overstreet, 1981; Katz *et al* 1990). Of the 300 million spermatozoa ejaculated only a few hundred actually reach the site of fertilization, the ampulla of the oviduct (Stone, 1993).

2.2 Fertilization

2.2.1 Capacitation of Spermatozoa

Before fertilization, the spermatozoa must undergo a period of maturation in the female reproductive tract for several hours; the time required varying in different species. These post fertilization events were first reported independently by Austin (1951) and Chang (1951) and the term "capacitation" was coined by Austin in 1952.

The precise definition of capacitation (Moore & Bedford, 1983) "is the process in the female reproductive tract (or in situ) that allows the acrosome reaction and the "activation" of spermatozoa otherwise known as hyper-activation, which promotes spermatozoa penetration of the zona pellucida" (Yanagimachi & Noda, 1970). Capacitation is thus a general term encompassing the biochemical and structural changes that occur in spermatozoa as they progress through the female reproductive tract. This process is still poorly understood and therefore difficult to define more precisely (Chang, 1984; Sidhu & Guraya, 1989). The topographical rearrangement of sperm surface components appears to include loss of a decapacitation factor (Chang, 1993; Eng & Oliphant, 1978) loss or migration of spermatozoa surface antigens (Myles & Primakoff, 1984), changes in lipid composition (Schwarz & Koehler, 1979) and the redistribution of lectin binding sites (Schwarz & Koehler, 1979).

Changes in sperm metabolism during capacitation lead to increases in oxygen uptake and glycolysis, and the expression of hyperactivated motility (Hamner & Williams, 1963). Before capacitation, spermatozoa swim with linear trajectories (Yeung & Wooley, 1984), but after hyperactivation, they move in a non-progressive circular manner (Suarez *et al.*1983) as the sperm head traces an erratic trajectory in response to increases in the amplitude and asymmetry of the flagellar wave form (Robertson *et al.*1988). Sub-fertile human sperm populations exhibit significantly lower levels of hyperactivation than their fertile counterparts and fewer spermatozoa bind to hemi-zona pellucida during IVF procedures (Coddington *et al.*1991).

In summary, capacitation enables the spermatozoa to undergo changes which lead to increased metabolism, the initiation of the acrosome reaction and the onset of hyperactivation. These processes enhance the ability of spermatozoa to bind to and penetrate the zona pellucida (Wassarman, 1987a; Sidhu & Guraya, 1989).

2.2.2 Acrosome Reaction (AR)

The name has a Greek origin meaning "sharp body" Popa, in 1927, first noticed that droplets of a 'sticky substance' were extruded from the tip of sea urchin spermatozoa when they were treated 'with egg water' (Popa, 1927 cited in Austin, 1968).The acrosome is a membrane-bound organelle that occupies the anterior region of the spermatozoa head, just above the nucleus and beneath the plasma membrane (Wassarman, 1987a). The acrosomal membrane that is nearest to the nuclear membrane is referred to as the inner acrosomal membrane and the one underlying the plasma membrane is called the outer acrosomal membrane. The AR is an exocytotic event involving focal fusion of the sperm plasma membrane with the outer acrosomal membranes at different sites and the formation of hybrid membrane vesicles. Initially the acrosomal matrix partially decondenses and the outer acrosomal membrane, alone or in combination with the plasma membrane, invaginates to form many vesicles in the acrosomal cap, many of which fuse with the plasma membrane leading to the fenestration of this structure. The membrane vesicles are finally discharged from the sperm head, thereby exposing the acrosomal contents and the inner acrosomal membrane (Wassarman *et al.*1986). The acrosomal vesicle is similar to a lysosome, and contains numerous hydrolases, such as hyaluronidase, proteinases, glycosidases, lipases and phosphatases (Allison & Hartree, 1970; McRorie & Williams, 1974; Morton, 1977). The AR requires extracellular calcium and is triggered by an influx of sodium and calcium and an efflux of hydrogen ions through the plasma membrane. The increase in intracellular pH appears to be due to an ATP-dependent H^+ pump (Endo *et al.*1991; Yanagimachi, 1981).

The place where the spermatozoa undergo the acrosome reaction remains controversial subject. Some workers have suggested that spermatozoa which spontaneously acrosome react *in vitro* are inferior cells which could be unable to

fertilize an egg *in vivo* (Schill *et al.*1988; White *et al.*1990). Infertile men who are polyzoospermic (with an increased number of spermatozoa in their ejaculates, usually > $350x10^6$/ml) have an abnormally high rate of spontaneously occurring acrosome reactions. This suggests that the place and timing of the acrosome reaction are important factors in determining the fertilizing ability of spermatozoa (Schill *et al.*1988; Tesarik, 1989). Some investigators have suggested that only capacitated acrosome-intact spermatozoa can penetrate the cumulus and bind to the zona pellucida (Storey *et al.*1984; Cherr *et al.*1986) and that both uncapacitated and acrosome-reacted spermatozoa are incapable of entering this extracellular matrix (Cherr *et al.*1986).

This general concept of the mechanisms regulating fertilization, in which capacitated spermatozoa with an intact acrosome penetrate the cumulus matrix, bind to the ZP, and only then undergo the AR, has received considerable attention (Florman *et al.*1984b; Wassarman, 1987a; Bleil & Wassarman, 1986; Leyton & Saling, 1989b). An interesting exception to this general rule is the guinea pig in which only acrosome reacted spermatozoa can bind to the ZP (Huang *et al.*1981), the acrosome intact spermatozoa lacking any affinity for the ZP even when capacitated. For most mammalian species the observations of Anderson *et al* (1975) seems to pertain: that both acrosome-intact spermatozoa and those that had undergone the acrosome reaction can penetrate the cumulus matrix but only the acrosome intact spermatozoa are able to bind to the ZP and fertilize the eggs. Other investigators (Storey *et al.*1984; Saling & Storey, 1979) have observed the same phenomenon using the chlortetracycline fluorescence procedure to score acrosome-intact and acrosome-reacted spermatozoa during *in vitro* fertilization procedures. Spermatozoa of the sea urchin, frog and other motile cells lacking hyaluronidase have been found to pass through the cumulus matrix of hamster eggs but were unable to bind to the ZP raising doubts about whether spermatozoa release hyaluronidase in order to penetrate the egg cumulus matrix.

Moreover, marsupial spermatozoa contain acrosomal hyaluronidase but their ovulated eggs are not surrounded by cumulus matrix (Morton, 1977; Talbot & DiCarlantonio, 1984), again indicating that it is not necessary for these cells to undergo the AR in order to reach the surface of the zona pellucida.

In most studies, the failure of spermatozoa to interact with the ZP appears to be at the level of ZP binding. If the ZP is removed from the oocytes, even spontaneously acrosome-reacted spermatozoa are able to fertilize an egg leading to the birth of normal young (Naito *et al.*1992). Under *in vitro* conditions, when cumulus-free (denuded) eggs were used, only acrosome-intact spermatozoa (Wolf & Inoue, 1976; Saling *et al.*1978; Saling & Storey, 1979; Phillips & Shalgi, 1980; Bleil & Wassarman, 1983) bound to the ZP and, after binding, the ZP induced the acrosome reaction (Saling *et al.*1979; Florman & Storey, 1982; Bleil & Wassarman, 1983). Using the scanning electron microscopy technique, Phillips & Shalgi (1980) observed that only acrosome-intact spermatozoa could be mechanically isolated from unfertilized hamster eggs, but the spermatozoa that had spontaneously undergone the AR failed to bind to ZP. In another study, using transmission electron microscopy, Florman & Storey (1982) showed that the percentage of acrosome-reacted spermatozoa on the zona surface reached 80% after 240 minutes post-insemination. Strong experimental evidence for an important biological role for the ZP has come from experiments in which acid or heat-solubilized ZP added to the spermatozoa are found to successfully induce the acrosome reaction (Florman & Storey, 1982; Bleil & Wassarman, 1983; Cherr *et al.*1986). In contrast acid-solublized embryos ZP failed to induce the spermatozoa to undergo the acrosome reaction (Florman *et al.*1984b). Similarly, pronase-treated ZP3 loses its ability to induce the acrosome reaction (Florman *et al.*1984b) suggesting that the intact polypeptide is essential for this function. Wassarman (1987a&b) subsequently indicated that both the polypeptide core and the

oligosaccharide side chains of ZP3 were required for the induction of the acrosome reaction, the peptide backbone forming a bridge, cros linking adjacent carbohydrate structures. The mechanism by which the sperm receptor ZP3 induces spermatozoa to undergo the acrosome reaction is thought to be a signal transduced exocytosis event involving guanine nucleotide binding proteins (Florman *et al.*1989; Endo *et al.*1987a; Leyton & Saling, 1989a). Activation of tyrosine kinase receptors as a consequence of ligand-induced aggregation is a widely known phenomenon and is in keeping with the apparent mechanism of action of ZP3. It is thought that ZP3 binding leads to clustering of receptors on the spermatozoa, which triggers acrosomal exocytosis. The signal transduction mechanism requires the involvement of a G protein to effect fusion between the plasma membrane and outer acrosomal membranes. A 95 kD protein present on the spermatozoa plasma membrane is involved in binding to ZP3, and appears to undergo an autophosphorylation event on binding the zona glycoprotein which, in turn, sets in motion a cascade of biochemical changes leading to exocytosis (Leyton & Saling, 1989a). A more detailed discussion can be seen later in chapter 2.3.12. A similar protein has been observed in human spermatozoa, the phosphorylation of which is enhanced by exposure to human and porcine zona proteins (Naz *et al.*1991a). Recently it has also been observed in the mouse that Gal-transferase on the sperm head mediates fertilization by binding oligosaccharide residues in the egg coat or zona pellucida. After the acrosome reaction the transferase relocalizes to a new membrane domain where it can no longer bind to ZP3, which is consistent with the inability of acrosome-reacted sperm to bind ZP3 or to initiate binding to the zona pellucida (Miller *et al.*1992). In view of the incidence supporting the involvement of both glycosyltransferases and tyrosin kinases in mediating sperm-zona interaction, we may conclude that the later is a highly complex event, which may involve several alternative pathways of gamete recognition and signal transduction.

2.2.3 Spermatozoa - Zona Pellucida Binding

The initial association between spermatozoa and the ZP is loose and is referred to as "attachment" (Hartmann *et al.*1972). The attached spermatozoa can be removed either by repeatedly pipetting the eggs (Hartmann *et al.*1972; Bleil & Wassarman, 1980a) or by dextran sulphate gradient centrifugation assays (Berger *et al.*1989). Later is the process of fertilization the spermatozoa become strongly bound to the zona pellucida, and are difficult to detach after repeated pipetting or gradient centrifugation, and this is called "binding" (Hartmann *et al.*1972).

2.2.4 Species Specificity

Lilly (Wassarman, 1990) was the first to observe that the interaction of complementary molecules on the surface of spermatozoa and eggs is essential for the process of fertilization. Fertilization is the most important event in the propagation of living beings and represents the co-ordinated process whereby haploid spermatozoa and egg fuse to form a diploid zygote which, in turn, is destined to become an adult organism. Specificity is essential, particularly where fertilization is external, for example in the sea, where the process takes place in an environment in which gametes from a number of different species are present. Sea urchin spermatozoa possess a lectin-like protein, called bindin, that interacts specifically with a glycoprotein receptor in the vitelline envelope of the sea urchin egg. Similar mechanisms also operate in ascidians and amphibians, as well as in plants. Mammalian fertilization takes place within the female reproductive tract, and in this situation, phenotype and behaviour help to maintain the species specificity of fertilization (Yanagimachi, 1978; quoted in Gulyas & Schmell, 1981) while gamete-specific receptor molecules resist cross-fertilization between closely related mammals. Fertilization involving heterologous gametes has occasionally been observed (Schmell & Gulyas, 1980; Bedford, 1977), for example,

mammalian hybrids such as the mule are created by cross-fertilization. Thus, it is generally accepted that mammalian fertilization is relatively, but not absolutely species-specific (Schmell & Gulyas, 1980). At the cellular level, the ZP plays a major role in preventing inter-species fertilization (Yanagimachi, 1977; Yanagimachi, 1984; Gulyas & Schmell, 1981; Adams, 1974). The most profound expression of this activity being in the golden hamster to oocytes of which will fuse with the acrosome reacted spermatozoa from a wide variety of species providing the zona pellucida has been removed (Yanagimachi, 1984).

2.2.5 Spermatozoa-Zona Pellucida Interaction

Once spermatozoa have traversed the cumulus matrix, the acrosome-intact cells bind to the zona pellucida. The sperm receptor molecule on the ZP is a glycoprotein, termed ZP3. This ZP3 glycoprotein not only serves as a recognition site for spermatozoa but also induces these cells to undergo the acrosome reaction (Mortillo & Wassarman, 1991). It is thought that O-linked oligosaccharides associated with the ZP3 molecule possess the sperm receptor activity (Bleil & Wassarman, 1988). Recent evidence involving the use of cross-linking agents in conjunction with mouse ZP3 have succeeded in identifying an Mr= 56000 sperm surface constituent which has been proposed as primary mouse-egg binding protein (Bleil & Wassarman, 1990). This protein (sp56) is located in the plasma membrane overlying the acrosomal region of acrosome intact mouse spermatozoa but is lost following the acrosome reaction. Proteolytic enzymes, particularly acrosin, released from the acrosome during exocytosis are thought to aid zona penetration (Gaddum & Blandau, 1970; McRorie & Williams, 1974; Gwatkin & Williams, 1977; Stambaugh & Smith, 1978). Specifically, acrosin aids the digestion of ZP1, a glycoprotein which serves a cross-linking function within the zona pellucida. The propulsive forces generated by sperm

movement also help spermatozoa to penetrate the ZP (Bedford & Roger, 1983). After the acrosome reaction has been induced the penetrating spermatozoa bind to another ZP glycoprotein, ZP2, which acts as a secondary binding site for spermatozoa (Bleil & Wassarman, 1986; Bleil *et al.*1988).

2.2.6 Spermatozoa-Egg Fusion

It is believed that an influx of Ca^{2+} ions into the acrosomal region during the acrosome reaction, is one of the changes that helps to modify the spermatozoa plasma membrane (Monroy, 1985) in preparation for sperm-oocyte fusion. In addition, local dehydration at the site of sperm-oolemma contact and other hydrophobic interactions might help mediate the fusion of the spermatozoa with the vitelline membrane of the eggs (Wilschut & Hoekstna, 1984). Once the fertilizing spermatozoon fuses with the egg plasma membrane, the sperm tail ceases to move (Yanagimachi, 1966; Gaddum Rosse, 1985) and the microvillous projections present on the egg, cluster tightly around and interdigitate over the sperm head (Schatten & Mazia, 1976). The cortex of the egg contains numerous cytoskeletal proteins such as actin and myosin, which produce contractile movements which help to draw the spermatozoon into the egg cytoplasm (Wassarman, 1987a).

Entry of more than one spermatozoa is lethal to the embryo and specific mechanisms have evolved for the prevention of polyspermy (Wassarman, 1987a; Bedford & Roger, 1983). One component of this process involves the discharge of the contents of the cortical granules into the perivitelline space (Gwatkin *et al.*1976; Whitaker & Steinhart, 1985) via mechanisms that are poorly understood. The contents of the granules induce both a 'zona reaction', leading to loss of sperm binding activity, and a hardening process, which makes this structure resistant to sperm penetration (ZP hardening). The zona reaction involves a modification of the sperm receptor ZP3, to a

less receptive form ZP3f, and a cros linking of the ZP, such that it becomes difficult to dissolve with reagents like proteinases and reducing agents (Bleil *et al.*1981; Wassarman *et al.*1986). Following fusion with the oocyte, the sperm head decondenses, the male pronucleus is formed, and the activated egg completes meiosis and the second polar body is released (Bedford & Roger, 1983). The entire sequence of oocyte growth and follicular maturation depends upon primary stimulation of the ovary by gonadotrophins, underpinned by local (paracrine and autocrine) levels of control emanating from within the follicle itself (Hillier, 1991).

2.3 Zona Pellucida

All mammalian eggs are surrounded by a 2-5 μm thick, transparent, extracellular matrix called the zona pellucida (ZP) which plays many roles during fertilization and early embryogenesis. As already discussed, the ZP contains species specific sperm receptors, furthermore, it induces the acrosome reaction and, following a signal transduced exocytotic event in the oocyte, blocks the entry of more than one spermatozoon (polyspermy) into the ooplasm. It also offers mechanical protection to the embryo during the preimplantation stages of pregnancy. Recent advances in biochemistry and molecular biology have helped us to understand the mechanisms by which the zona pellucida plays its many roles.

The mouse ovary has 10,000 to 15,000 primordial oocytes at birth and these non-growing oocytes do not have a visible ZP. During the oocyte growth phase (20-40μm diameter) the ZP first appears as a thin, diffuse envelope after about 2 weeks and later as a thick, dense coat when the oocytes have reached 60-85 μm in diameter (Wassarman & Josefowicz, 1978). In the following section, we shall examine the

information that is available on the structure and composition of this important biological (ZP) structure.

2.3.1 Zona Pellucida Synthesis

The site of production of the ZP has been a controversial issue for a long time. Many of the non-mammalian egg coats are not synthesized by oocytes but by their surrounding follicle cells or nurse cells (Dumont & Brummet, 1985). Under *in vitro* conditions, when denuded mouse oocytes (free of follicular cells) are cultured with either ^{35}S-methionine or ^{3}H-fucose, the oocytes can synthesise all the major ZP glycoproteins, in contrast the follicular cells are not able to synthesise any ZP components (Wassarman, 1988b; Bleil & Wassarman, 1980b). In vitro culture experiments also suggest that three of the sulphated glycoproteins of the ZP are synthesized by the oocytes (Shimizu *et al.*1983). A specific antiserum directed against mouse ZP2 could precipitate ZP2 from the oocytes only, not from the follicular cells (Greve *et al.*1982). On the contrary, Wolgemuth *et al* (1984) used an anti-ZP antiserum to immunolocalize ZP synthesis, and have reported immunostaining of ZP in the cytoplasm of oocytes and within the cytoplasm of the follicular cells. With the arrival of molecular probes, *in situ* hybridisation studies have clearly shown that, ZP3 mRNA is only expressed in oocytes and not in follicular cells (Wassarman, 1989; Liang *et al.*1990; Philpott *et al.*1987).

2.3.2 ZP Biochemical Characteristics

The ZP of mouse oocytes consists of three different glycoproteins namely ZP1 with apparent molecular. mass (Mr) of 200,000, ZP2 (Mr=120,000) and ZP3 (Mr=83,000) (Wassarman, 1987a; Wassarman, 1988a; Wassarman, 1990). Like other secreted glycoproteins, the zona pellucida shows considerable micro heterogeneity expressed in

the presence of multiple charge isomers. The isoelectric point of ZP1 is 4.1; ZP2 5.2; and ZP3, 4.2-5.2, so that all three glycoproteins are acidic in nature (Greve *et al.*1982; Salzmann *et al.*1983). All of the mouse glycoproteins contain sulphated oligosaccharide complexes. When run on SDS-PAGE, ZP2 and ZP3 migrate as monomers but ZP1 behaves as a dimer (Wassarman, 1988b; Wassarman, 1990). Under reducing conditions ZP1 co-migrates with ZP2 on a one dimensional gel with molecular mass of 120,000 whereas ZP3 is slightly retarded, suggesting that the native form of ZP3 possesses intramolecular disulphide bonds. Endo-ß-N-acetyglucosaminidase has been used to digest the mouse glycoproteins although this enzyme was found to be inefficient, implying that all ZP glycoproteins have aspargine-linked (N-linked) complex type oligosaccharides. Under *in vitro* culture conditions, mouse oocytes fail to "core" glycosylate all of the mouse zona proteins, in the presence of the antibiotic tunicamycin an inhibitor which prevents N-linked glycosylation (Roller & Wassarman, 1983). The molecular mass of ZP2 and ZP3 decreases if alkaline-ß-elimination is carried out in the presence of ^{3}H-$NaBH_4$. This is due to the removal of oligosaccharides that have ^{3}H-N-acetylgalactosaminitol at the reducing termini (Bleil & Wassarman, 1988). The relatively low isoelectric points of the mouse ZP glycoproteins are due to the presence of the oligosaccharide side chains, such that these molecules are acidic in the glycosylated form but become more basic if treated with endo F or neuraminadase. The sialic acids on the glycosaccharides of the ZP glycoproteins are the major contributors to their acidic nature. In vitro studies have shown that ZP from oocytes grown in the presence of tunicamycin and subsequently digested with EndoH yielded ZP3 species of Mr=81,000 and 44,000 (Salzmann *et al.*1983). Similar molecular masses were obtained after complete deglycosylation with trifluoromethane sulphonic acid (TFMS), a reagent that removes both N-and O-linked oligosaccharides from glycoproteins. All ZP glycoproteins are N-glycosylated

posttranslationally in the endoplasmic reticulum to give rise to 160,000, 91,000, 56,000 and 53,000 molecular weight species, all of which possess high mannose-type N-linked oligosaccharides. ZP2 has six N-linked and ZP3 either three or four N-linked oligosaccharides per molecule (Greve *et al.*1982). Successful secretion of ZP2 requires addition of N-linked oligosaccharides but these are not required for ZP3 secretion, under *in vitro* culture conditions. The nascent N-linked oligosaccharides of the ZP are converted to complex-type oligosaccharides, and, at the same time nascent ZP2 and ZP3 obtain their O-linked oligosaccharides and these mature ZP glycoproteins are secreted into the extracellular space (Salzmann *et al.*1983).

2.3.3 Porcine ZP Glycoproteins

Porcine ZP is composed of four glycoprotein families of molecular mass 80,000-90,000 (ZP1), 60,000-65,000 (ZP2), 55,000 (ZP3), 20,000-25,000 (ZP4) (Hedrick & Wardrip, 1986; Yurewicz *et al.*1987; Dunbar & Raynor, 1980; Henderson *et al.*1987b). Like the mouse, porcine ZP glycoproteins are acidic in nature, with pIs in the range 5.2 to 5.6 pH units. Work from different laboratories suggests that ZP2 and ZP4 are derived from ZP1 by proteolysis and reduction of its intermolecular disulphide bonds. Evidence for this comes from the fact that the total sum of the amino acids and carbohydrates in ZP2 and ZP4 is equal to those in ZP1 (Hedrick & Wardrip, 1987). ZP1 and ZP2 have similar peptide domains and some peptide segments share an identical primary structure. ZP1 is immunologically related to ZP2 and ZP4, but ZP2 and ZP4 are not immunologically related to one another (Hedrick & Wardrip, 1987). On one-dimensional gels under non-reducing conditions, porcine ZP gives rise to 80,000-90,000 and 55,000 molecular mass components. The porcine ZP3 band contains two distinct glycoprotein species namely ZP3å and ZP3ß. The 80,000-90,000 species corresponds to ZP1 and the heterodimer of ZP2 and ZP4.

Deglycosylation of porcine ZP3 with TFMS yields two polypeptides of Mr=34,000 and 36,000 and these polypeptides differ from one another in their primary structure as determined by amino-terminal sequence analyses and peptide mapping experiments, as well as immunologically (Yurewicz *et al.*1987). The average amino acid composition of these two polypeptides is identical to that of purified ZP3. Partial deglycosylation of ZP3 with endo-ß-galactosidase yields two glycoprotein types of Mr=42,000 and 46,000. They differ in their amino acid and carbohydrate composition, peptide map amino-terminus analyses, and immunoreactivity. This implies that porcine ZP3 is composed of two structurally and immunologically different lactoseaminoglycan-containing glycoproteins, representing different genes (Yurewicz *et al.*1987). Henderson *et al* (1987b) have demonstrated that chemical deglycosylation of porcine ZP using trifluoromethanesulphonic acid (TFMS) resulted in the production of five discrete protein bands on one-dimensional sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) with molecular masses of 66(ZP1), 52(ZP2), 36(ZP3 å) 32(ZP3ß), and 16 kD. All the above porcine ZP components were purified to homogeneity using one-dimensional SDS-PAGE and the antibodies generated to the 32 kD deglycosylated polypeptide inhibited human gamete interaction (Henderson *et al.*1987a).

2.3.4 Hamster ZP

Under non-reducing SDS-PAGE the hamster ZP migrates as two bands having Mr=208, 000 (range, 127-288,000) and 56,000 (range 54-58,000) (Moller *et al.*1990). HPLC analysis also reveals two bands of similar size, but when these HPLC-fractionated samples are analysed by SDS-PAGE a further band of Mr=103,000 is revealed (range 92-113,000). Hamster ZP1 consists of two polypeptides of 103,000, held together by intermolecular disulphide bonds and cannot be distinguished from

hamster ZP3 on the basis of size (Moller *et al.*1990). In the presence of reducing agents, the Mr= 200,000 hZP1 molecule migrates on SDS-PAGE with an apparent Mr=103,000. This suggests that hZP1, like mouse ZP1, is composed of two polypeptides held together by intermolecular disulphides.

2.3.5 Human ZP

Less information is available on human ZP because of the paucity of material. Human ZP after heat solublization, and iodination and SDS-PAGE under non-reducing conditions yields bands migrating in the range 92-12,000 (ZP1, 2) and 57-73,000 (ZP3). In the presence of a reducing agent human ZP separates into molecules of Mr=97,000 (ZP1), 82,000 (ZP2), and 70,000 (ZP3). It has also been noticed that there is also a substantial disappearance of ZP1 from fertilised eggs, when compared with unfertilised eggs under reducing condition (Shabanowitz & O'Rand, 1988b).

2.3.6 Structure of the Zona Pellucida

Using a variety of techniques including light and electron microscopy and different probes such as lectins, antibodies and ruthenium red it is possible to gain a limited understanding of the structure of the ZP. The ZP appears a meshwork of randomly arranged fibrillogranular strands. The binding sites for ruthenium red, lectins and antibodies lie at the exterior rather than at the interior of the ZP. Some of the reagents used could not penetrate the interior of the ZP. However, some evidence suggests that the strands, or fibres, are more densely packed on the internal surface of the ZP (Dietl & Czuppon, 1984). Isolated ZP solubilized with elastase at pH 5.5 produce primarily large complexes consisting of interconnected or branched filaments in which heterologous interactions between ZP1, ZP2, and ZP3 are observed. Treatment with chymotrypsin and dithiothreitol reveals primarily individual or unbranched filaments

in which heterologous interactions, at least between ZP2 and ZP3, are maintained. ZP filaments exhibit a structural repeat of about 14-15nm in the electron microscope. ZP2:ZP3 is present as a heterodimer every 14 nm and this was demonstrated in both isolated filaments and intact ZP by SDS-PAGE and by electron microscopy using monoclonal antibodies specific to ZP2 and ZP3. The SDS-PAGE experiments also revealed that the ZP2:ZP3 dimer has a Mr of approximately 180, 000. Treatment of ZP with dithiothreitol and chymotrypsin results in reduction and limited proteolysis of ZP1 and the solubilized preparation contained filaments that were no longer interconnected. This suggests that ZP1 serves as an interconnection between ZP2:ZP3 giving rise to a three-dimensional matrix (Greve & Wassarman, 1985).

2.3.7 Zona Pellucida Genes (Mouse)

Analysis of genomic DNA (Kinloch *et al.*1988; Chamberlin & Dean, 1989) has revealed that the mouse ZP3 gene is 10kb in length with 8 exons and is located on mouse chromosome number 6. The sizes of the mouse exons are 338 bp, 119 bp, 104 bp, 184 bp, 118 bp, 92 bp, 137 bp, and 225 bp for exons 1-8, respectively. The introns range from 2520 bp to 195 bp. In the 5' flanking upstream region there is a TATA box 29 nt from the transcription start site. Also in the upstream region there is a short region of internal homology at approximately -220 to -320 from the first exon. Many copies of a 54 consensus sequence are found as tandem arrays in the 5'-flanking regions and the cis-acting elements in mouse ZP3. Another tandem array is also present beginning 43 bp, 3' to the polyadenylation site at the 3' end of the gene. This sequence contains 12 nucleotides (GGTGGGATTGGT) perfectly repeated 11 times. A startling feature of the ZP3 gene is that the stop codon, TAA, is buried in the polyadenylation signal, AATAAA (Chamberlin & Dean, 1989; Kinloch *et al.*1990).

Mouse ZP3 cDNA is 1317 nt in length and has a poly(A) tail of 200-300nt (Ringuette *et al.*1986; Ringuette *et al.*1988). There is a 29 nt 5' untranslated region followed by a single open reading frame, giving rise to a nascent polypeptide of Mr= 46,307. The N-terminus has a 22 amino acid signal sequence, leaving a core polypeptide of Mr=43,943. It has 13 cysteine, 29 proline, 70 serine plus threonine, 13 histidine, 6 tryptophan, and 2 methionine residues (Kinloch *et al.*1988; Ringuette *et al.*1986; Ringuette *et al.*1988). ZP3 contains a high number of proline and serine plus threonine residues. There are 6 potential glycosylation sites for N-linked oligosaccharides, 3 or 4 of which are of the complex-type. The hydrophobicity plot shows that ZP3 is neither strongly hydrophobic nor hydrophilic, except for 2 small domains near the carboxyl terminus (Dean *et al.*1989). The ZP3 gene is expressed exclusively in actively growing oocytes. Zona transcripts cannot be detected in the oocytes (10-15μm) of primordial follicles, and maximum levels are observed in 50μm-diameter oocytes that contain 1 ng of ZP2 transcripts and 0.4 ng of ZP3 transcripts representing 1 and 80%, respectively, of total poly(A^+) RNA (Testart & Mendoza Oltras, 1986; Philpott *et al.*1987; Roller *et al.*1989; Schickler *et al.*1992). Upon meiotic maturation the levels of ZP2 and ZP3 mRNA drop to 5% of the peak levels of ZP transcripts (Liang *et al.*1990). Secondary structure prediction methods indicate that ZP3 contains little å-helical-forming potential. All the N-linked glycosylation sites are situated in the hydrophilic stretches, which are predicted to form reverse turns or coils.

In female, mammals zona pellucida genes are the only known oocyte and therefore sex-specific genes expressed in mammals. ZP3 gene expression is found in growing oocytes of 2-3 weeks of age in the mouse. The cis-acting element that governs expression of the ZP3 gene in oocytes is reported to be present within 6.5 kb in the upstream region (Lira *et al.*1990). This has been demonstrated in transgenic

mice by introducing the firefly reporter gene along with the 6.5 kb upstream of the ZP3 gene. Recently, it has been demonstrated that the cis-acting element responsible for oocyte-specific expression in transgenic mice is located within the first 470 bp of the mouse ZP3 (mZP3) promoter. Extracts of mouse ovaries contain a Mr=~60,000 protein, that binds specifically to the sequence 5'--GATAA-3' located within the first 100bp of the mZP3 promoter. This protein is known as OSP-1, and is present only in growing oocytes but not in ovulated eggs or two-cell embryos (Schickler *et al.*1992). The mouse and human ZP2 and ZP3 genes 5'--flanking region contains five short DNA sequences I, IIA, IIB, III, and IV (4 to 12 bp) that are 60-100% identical and are approximately equidistant upstream of the TATAA box in the four genes. Deletion and mutational analysis of the five short 5'-flanking regions of ZP3 revealed that element IV is a 12-bp sequence that plays a crucial role in directing expression in oocytes. The element IV (CACG/CTG) forms protein-DNA complexes of indistinguishable mobility with lysates from oocytes and not from any other tissues (Millar *et al.*1991).

2.3.8 Human ZP3 Gene

The human genomic DNA is a 18.3 kb transcription unit, which is double the size (8.6-kb) of the mouse gene. The human ZP3 gene is composed of 8 exons spanning a length of 18.3 kb. The exons range from 92 to 312 bp, which is almost identical in size to the eight exons of mouse ZP3 and the nucleotide sequence is 74% similar to the mouse. There are at least 16 human Alu repeat sequences present at the locus of the human ZP3 homologue. The human and mouse ZP3, in the first 250 bp upstream region has four similar elements of 8-15 bp, which are 82-90% conserved. Like the mouse the human ZP3 has short untranslated regions in the 5' and 3' regions. The tandem repeat found upstream of the transcription start site of mouse ZP3 and the

perfect 12-bp repeat found 3' to the gene are not present in the human gene (Chamberlin & Dean, 1989) Human ZP3 involves a single open reading frame of 1272 nt that encodes a 424 amino acid protein. The amino acid sequence is 67% similar to the mouse ZP3 and the predicted secondary structure is also conserved. The human ZP3 polypeptide chain contains four potential N-linked glycosylation sites, three of which are conserved in the mouse, and 66 potential O-linked glycosylation sites in human ZP3, 71% of which are conserved in the mouse. All of the 13 cysteine residues found in the human ZP3 are conserved in the mouse protein, suggesting that the secondary structures of these proteins are preserved. The region that is very dissimilar from the mouse ZP3 lies in the carboxyl region and corresponds to the hydrophilic peaks that presumably lie on the surface of the protein (Chamberlin & Dean, 1990). Interestingly the human ZP3 is not a single-copy gene in that human genome has second polymorphic locus which, due to an extra G residue in exon 8, has the potential to encode a truncated protein of 372 amino acids. Direct sequence analysis of polymerase chain reaction-amplified exon 8 DNA of 56 individuals of various human populations revealed three different sequence patterns: one containing only ZP3-424-coding sequences and two containing ZP3-424- and ZP3-372-coding DNA (van Duin *et al.*1992).

2.3.9 Hamster ZP3 Gene

The hamster genomic DNA is 7900 bp in length and consists of eight exons and seven introns. Hamster mRNA contains 1266 nt of coding sequence, 6 nt less than mouse RNA (Kinloch *et al.*1990). Like the mouse and human ZP3 mRNAs the hamster ZP3 mRNA 5'- and 3'-noncoding regions are very short, compared with most eukaryotic mRNAs (Kozak, 1984). Ten nucleotides around the ATG initiation codon of hamster (hZP3) match those of a consensus sequence (GCCGCCA/GCCATGG) reported to be

involved in translation initiation in vertebrates (Kozak, 1987) and the termination codon TAA forms part of the poly(A) addition signals, AATAAA (Proudfoot & Brownlee, 1976). Many copies of a 54 consensus sequence are found as tandem arrays in the 5'-flanking regions and the cis-acting elements known to be present in mouse ZP3 are also present in hZP3, which is approximately 85% similar (Kinloch *et al.*1990). The predicted amino acid sequence of hZP3 is 81% identical to mouse ZP3, and hZP3 is similarly characterised by a high number of serine plus threonine residues. Hamster ZP3 also has 13 cysteine residues, as has the mouse ZP3, in conserved locations. Hamster ZP3 has four potential N-glycosylation sites and, three of which are conserved between hZP3 and mZP3. Secondary structure prediction methods indicate little å-helix-forming potential; rather, it consists of reverse turns or coils (Kinloch *et al.*1990) and the hydropathicity plot is virtually identical with that generated by mouse ZP3 (Kyte & Doolittle, 1982).

2.3.10 Recombinant ZP Proteins

The hamster and the mouse ZP3 genomic DNA has been cloned in pKJ-1, which contains the bacterial neomycin resistance gene placed between the mouse phosphoglycerate kinase-1 (pgk-1) 5'- and 3'- regions. The mouse and the hamster genomic DNA was therefore under the control of the phosphoglycerate kinase-1 promoter (Kinloch *et al.*1991). The embryo carcinoma cell lines transfected with a genomic construct of mZP3 and hZP3 secreted large amounts of recombinant ZP3 glycoproteins. The molecular mass of the secreted mZP3 and hZP3 glycoproteins were Mr=83,000 and Mr=49,000 respectively. The embryonic cell line of mouse origin produced recombinant mouse ZP3 of the right size (Mr=83,000), whereas the recombinant hamster protein was Mr=49,000 as compared to the wild type, which has a Mr value of 56,000. Kinloch *et al* (1991) also demonstrated that the recombinant

mZP3 is biologically active as a sperm receptor *in vitro*, but hZP3 is not. Similarly the mZP3 could induce the acrosome reaction in murine spermatozoa, but the hZP3 could not. It has also been demonstrated that the embryonic cell line transfected with mZP3 can bind murine spermatozoa *in vitro* (Kinloch *et al.*1991). Beebe et al (1992) have shown that the full length mouse ZP3 cDNA construct cloned in cytomegalo virus and transfected into the mouse L-929 cell line, produced recombinant mouse ZP3 with a relative molecular mass of 60,000-70,000. Another construct cloned into the vaccinia virus promotor and transfected into the green monkey CV-1 cell line, also produced mouse recombinant ZP3 of the same size. The recombinant glycoproteins were biologically active since they inhibited spermatozoa binding and induced the acrosome reaction (Beebe *et al.*1992).

2.3.11 ZP3 : the Sperm Receptor

Respective complementarity between molecules on the surface of gametes ensures species specific interactions during fertilization. At present there appears to be a number of different candidates for a sperm zona-binding protein that interacts with zona pellucida. These comprise enzymatic molecules like galactosyltransferase (Lopez *et al.*1985; Lopez & Shur, 1987; Miller *et al.*1992; Fayrer Hosken *et al.*1991) the acrosomal enzyme acrosin (Moos *et al.*1990; Topfer Petersen *et al.*1990; Aitken *et al.*1990; Jones, 1991) and a trypsin-like protease in the mouse (Leyton & Saling, 1989a). The egg's receptor, on the other hand, appears to be one or more carbohydrate moieties of the ZP molecules, ZP3 and ZP2, at least in the mouse (Wassarman *et al.*1986; Bleil & Wassarman, 1986; Bleil & Wassarman, 1990; Bleil *et al.*1988; Vazquez *et al.*1989; Wassarman, 1989; Florman & Wassarman, 1985). In the pig, ZP3 of Mr=55,000 has been identified as the boar sperm receptor (Sacco *et al.*1989; Peterson *et al.*1991; Peterson & Hunt, 1989). Further research has shown that

Mr=90,000 and Mr=55,000 (å) components have the capacity to bind to sperm populations (Berger *et al.*1989), whereas in the rabbit ZP2 and ZP3 have been shown to interact with spermatozoa (O'Rand *et al.*1988). Impressive studies by Bleil and Wassarman (1986) have shown that in the mouse, purified ZP3 binds exclusively to the sperm head only and not to any other parts of spermatozoa and even at low concentration, ZP3 prevents the binding of spermatozoa to ova *in vitro*, whereas a variety of other non-specific glycoproteins have no effect on this process (Bleil & Wassarman, 1980d; Florman & Wassarman, 1985; Bleil & Wassarman, 1988; Leyton *et al.*1989). Only acrosome intact spermatozoa are able to bind to the unfertilised egg ZP (Bleil & Wassarman, 1986; Mortillo & Wassarman, 1991). It has been shown that acrosome-intact spermatozoa bind to silica beads coated with ZP3 and undergo the acrosome reaction but spermatozoa do not bind to other zona glycoproteins such as ZP2 coupled to the silica beads (Vazquez *et al.*1989). In the mouse, ZP3 at a very low concentration is able to induce the AR (spermatozoa exocytosis) *in vitro* but no other ZP glycoproteins can (Kopf, 1989; Leyton & Saling, 1989b; Leyton *et al.*1989; Endo *et al.*1987b; Endo *et al.*1987c). ZP3 from fertilised eggs is unable to bind to spermatozoa (Bleil & Wassarman, 1986) or to induce the acrosome reaction (Bleil & Wassarman, 1983) suggesting that ZP3 undergoes biochemical changes once spermatozoa is bound and thus prevents further entry of spermatozoa (block to polyspermy).

Over the past decade, the detailed biochemical structure of the sperm receptor has been studied extensively (Florman & Wassarman, 1985; Oikawa *et al.*1973; Huang *et al.*1982; Ahuja, 1982; Ahuja, 1985; Shur & Hall, 1982). In the mouse zona, Florman and Wassarman (1985) identified a glycopeptide from the ZP3 molecule with an O-linked carbohydrate moiety that was responsible for the binding of the spermatozoa to the zona pellucida. After extensive digestion with pronase, ZP3

generated a population of relatively small glycopeptides (about Mr=1500-6000) which still retained their sperm receptor activity but were unable to induce the acrosome reaction (Florman *et al.*1984a; Wassarman, 1989; Leyton & Saling, 1989b). ZP3 loses its sperm receptor activity when chemically deglycosylated with trifluoromethane sulphonic acid which removes both N-linked and O-linked oligosaccharides (Edge *et al.*1981) or when O-linked oligosaccharides are selectively removed from ZP3 by mild alkaline hydrolysis (ß-elimination; Sharon, 1975; Wassarman *et al.*1985; Wassarman *et al.*1989). On the contrary, selective removal of N-linked oligosaccharides from ZP3 had no significant effect on ZP3's ability to serve as a sperm receptor (Wassarman *et al.*1985; Wassarman, 1989). When ZP3 was subjected to ß-elimination in the presence of sodium borohydride, the result was the release of O-linked oligosaccharides having N-acetyl-D-galactosaminitol at their reducing termini (Florman & Wassarman, 1985). Furthermore, O-linked oligosaccharides fractionated by HPLC having an apparent molecular mass between 3400-4600, possessed sperm receptor activity. Studies have shown that when such O-linked oligosaccharides were treated with different exoglycosidases only å-galactosidase and å-fucosidase have a significant effect (Bleil & Wassarman, 1988; Wassarman, 1989). The involvement of a terminal galactose has also been suggested to be important for sperm receptor activity. Sperm receptor activity was lost when ZP3 was treated with galactose oxidase which converts the C-6 position alcohol of terminal galactose and N-acetylgalactosamine residues of ZP3 oligosaccharides to an aldehyde. The sperm receptor activity was restored by regeneration of a C-6 position alcohol on galactose by reduction with sodium borohydride (Bleil & Wassarman, 1988).

A new concept has been put forward for sperm-zona interaction in the mouse involving a sperm surface ß-,1,4-galatosyl transferase (Gal-transferace) that behaves like a lectin, binding to a specific glycoside substrate on ZP3 (Miller *et al.*1992). The

specificity of this interaction is remarkable since the sperm galactosyl transferase binds to ZP3 but not to any of the other zona pellucida glycoproteins. If the Gal-transferase binding sites on ZP3 are blocked by incubation with purified Gal-transferase and UDP-galactose, then ZP3 loses its biological activity. Removal of the Gal-transferase binding sites with N-acetyl hexoseaminidase was also found to destroy the ability of ZP3 to behave as a sperm receptor, while treatment with å-galactosidase had no effect, in contrast to the report of Bleil and Wassarman (1988). After the acrosome reaction, spermatozoa lose their affinity for ZP3 and the Gal-transferase becomes relocated to a new membrane domain and loses its reactivity towards ZP3 (Miller *et al.*1992). In contrast to the above results Noguchi *et al.*(1992) have shown that neutral carbohydrate chains derived from the N-linked ZP oligosaccharides are responsible for the sperm receptor activity expressed by porcine ZP3. Fucoidin, blocks fertilization in a number of species like hamster, human, boar, rat and mouse and disrupts the binding of acrosome-reacted guinea pig spermatozoa to homologous zonae pellucidae(Huang *et al.*1982; Ahuja, 1982). Fucoidin and ZP glycoproteins are both known to bind to acid-extracted proteins from guinea pig spermatozoa in the western blot detection system (Jones & Lancaster, 1988).

In the porcine model, a 55kD (ZP3) glycoprotein was found to possess sperm receptor activity (Sacco *et al.*1984). Digestion of ZP3 with endo-ß-galactosidase yields two glycoprotein species of 42,000 (ß)and 46,000 (å)molecular mass, which differ in their amino acid and carbohydrate composition, and have immunologically distinct lactosaminoglycans (Yurewicz *et al.*1987). Sacco *et al* (1989) have presented evidence that the ZP3å (46,000 kD) glycoproteins of MR=55,000 protein possess sperm receptor activity and have shown that the carbohydrate residue plays a critical role in sperm receptor function. In the porcine model purified O-glycans inhibited

sperm binding, but was 2-3 orders of magnitude less effective than ZP3 as a competitive ligand (Yurewicz *et al.*1991).

It has been reported that mouse ZP2 serves as a secondary sperm receptor and that it binds exclusively to the inner acrosomal membrane of acrosome-reacted spermatozoa and that acrosin might be its ligand (Bleil & Wassarman, 1986; Bleil *et al.*1988; Mortillo & Wassarman, 1991). Since the sperm Gal-transferase possesses no affinity for ZP2 it is unlikely that this particular sperm surface entity is involved in secondary binding phenomena. The most likely candidate for the ZP2 receptor at the present time is (pro) acrosin, an acrosomal constituent than expresses, in addition to its inherent proteolytic activity, a fucose binding domain that is capable of interaction with the zona pellucida. During the acrosome reaction, the alkalinization of the acrosomal contents leads to the autoactivation of proacrosin to generate acrosin through the modification of both the N-terminal and C-terminal ends of the protein (Fock-Nuzel *et al.*1984; Cechova *et al.*1988; Baba *et al.*1989; Topfer Petersen *et al.*1990). Acrosin is liberated from the acrosomal vesicle during a very early stage of the acrosome reaction and apparently becomes bound to the outer surface of the plasma membrane, at least in the human (Tesarik *et al.*1988). The relocation of proacrosin/acrosin on the sperm surface places this molecule in an ideal position to mediate the secondary phase of sperm-zona interaction following the initial attachment of the sperm plasma membrane to the zona surface through the mediation of ZP3. The pig has been used as an animal model for these studies and the results indicate that, as long as protease inhibitors are present in the incubation medium, then the major zona-binding entity in porcine spermatozoa is an Mr=53000 molecule with all the features of proacrosin (Topfer Petersen & Henschen, 1987). The evidence for ZP2 binding on proacrosin/acrosin is not very strong, but studies have suggested that basic residues

like sulphate groups along the polymer backbone on proacrosin/acrosin are likely determinants (Jones, 1990).

2.3.12 ZP3 as Acrosome Inducer

For successful fertilization to occur, spermatozoa must recognize and bind to the ZP. ZP-binding triggers exocytosis in the acrosomal region of the sperm head, which is mandatory for sperm passage through the ZP and access to the plasma membrane where gamete fusion occurs (Bunch *et al.*1992). Purified ZP3 induces mouse spermatozoa to undergo the AR *in vitro*. However, a higher concentration of ZP3 is needed for acrosome induction than for prevention of sperm-zona binding (Bleil & Wassarman, 1983). Proteolytic fragments of ZP3 are known to retain the specific binding, but not the acrosome reaction-inducing capacity of native ZP3, suggesting that the polypeptide core of ZP3 is necessary for the acrosome reaction induction (Florman *et al.*1984a; Wassarman, 1989). Recently it has been confirmed, using gold-labelled zona pellucida glycoproteins under the transmission electron microscope, that mouse ZP2 and ZP3 bind preferentially to the sperm head of acrosome reacted and acrosome intact spermatozoa, respectively(Mortillo & Wassarman, 1991). Recent studies suggest that induction of the AR by ZP3 could depend on multivalent interactions with the sperm surface leading to aggregation of receptors for ZP3 in the sperm plasma membrane. This was demonstrated by incubating mouse spermatozoa with small ZP3 glycopeptides that were unable to induce these cells to undergo the AR *in vitro* until the glycopeptides had been cross-linked on the sperm surface with specific antibodies (Leyton & Saling, 1989b). Investigators have (Leyton & Saling, 1989a) identified a Mr=95000 protein (p95) as a putative ZP3 receptor. According to these authors bound multivalent ZP3 facilitates the oligomerization of p95 and leading to the autophosphorylation of this putative receptor on tyrosine residues, initiating the

intracellular cascade that finally results in acrosomal exocytosis. To date, no experiments have been performed with tyrosine kinase inhibitors to determine whether such reagents have the capacity to interfere with zona-induced acrosome reactions.

2.4 Immunocontraception

People of diverse religion and culture regard immunology with high esteem, so that they willingly accept vaccination and other immunological treatments (Tyler, 1961). In that context, immunocontraception may be an alternative to the existing methods of contraception. An ideal immunocontraceptive must be one that is specific to a particular antigen, is without any untoward effects, is cheap enough to produce, and possibly, though not necessarily, reversible in nature.

Metchnikoff (1899) injected guinea pigs with the semen or the macerated testes of man, bulls, guinea pigs and rabbits. He found that the serum of these different animals agglutinated and immobilized the spermatozoa of all these various species. There are a number of investigators who have actively immunized women with intact spermatozoa or extracts of human, bull or ram spermatozoa, and all have obtained positive results (Baskin, 1935; Laffont & Theron, 1934; Escuder, 1936). The contraceptive potential of antibodies against fertilisin, prepared from the surface of the sea-urchin egg, has also been demonstrated to block further development of treated eggs (Tyler, 1957). There are primarily three potential candidate antigens which are currently under development for immunocontraceptive purposes 1) human chorionic gonadotrophin (hCG), 2) the zona pellucida and 3) sperm surface antigens.

Human chorionic gonadotrophin is secreted by the early embryo and can be detected as early as 72 hours post implantation. This hormone is involved in the maternal recognition of pregnancy through the rescue of corpus luteum function

during the earliest stages of pregnancy. In this way, the embryo prevents the cyclical demise of the corpus luteum that precipitates menstruation. There is considerable homology in the primary amino acid structure of hCG and the pituitary gonadotrophin LH (luteinizing hormone). The å-subunits of the two hormones are identical and the ß-subunit, has an additional short sequence of 37 amino acid at the carboxyl terminus of hCG (Mitchison, 1990). Stevens and Crystle (1973) actively immunized post-menopausal and sterilized pre-menopausal women with sulphonic derivatives of whole hCG and suggested that hCG could be made immunogenic in humans. Vaccines incorporating the carboxyl sequence, conjugated to immunogenic carriers such as diptheria toxoid have been used in phase I clinical trials and have shown to produce antibody titres in the absence of any detectable side effects (Stevens *et al.*1990). An alternative approach employed the whole ß-subunit of the hCG molecule as the antigen. Initial studies were based on the conjugation of the ß-subunit of hCG to tetanus toxoid. This study had demonstrated that it was possible to raise antibodies against hCG and that the response was reversible and free from any harmful side-effects (Talwar *et al.*1988; Talwar *et al.*1990). In order to enhance the immunogenicity of hCG, a chimeric hCG molecule has been created, in which the ß-subunit has been annealed to the å-subunit of ovine LH. This approach had generated high anti-hCG antibody titres in many of the women involved in phase 1 clinical trials in India, without any untoward side effects (Gaur *et al.*1990).

Studies have shown that 5% of patients attending infertility clinics appear to be infertile as a result of auto- or iso-antibodies against spermatozoa (Aitken *et al.*1992). These patients appear to suffer no other consequence of their immunity apart from their infertility, the feasibility of developing a safe, effective contraceptive vaccine based on sperm surface antigens would appear to have been demonstrated. It has also been shown that patients having antisperm antibodies according to the conventional

sperm-agglutination assays appear to generate a broad spectrum of antibodies directed against multiple antigenic targets, and not to single dominant surface determinants (Aitken *et al.*1987). Surface antigens might not be integral components of plasma membranes or acrosomal enzymes, since they may be lost during capacitation. The ideal antigen should perhaps be present on the spermatozoa surface as well as inside the cell (Kurpisz, 1990). A sperm surface antigen of internal origin which had received wide attention is SP10. A monoclonal antibody against this antigen cross reacts only with testicular germ cells and mature spermatozoa and not with somatic cells (Herr *et al.*1990). A 95 kD antigen has also been reported (Moore *et al.*1987) which is located intracellularly and when targeted by a monoclonal antibody disrupts the attachment of human spermatozoa to the zona pellucida. Other antigens derived from the acrosomal region of spermatozoa, termed HS-11 and HS-63 with Mr=35,000-50,000 and Mr=40,000-50,000 respectively, have also demonstrated contraceptive potential since antibodies against them interfere with the acrosome reaction induced by either the ionophore, A23187, or ZP. A well characterised sperm surface antigen of guinea pig origin called PH20 consistently produces a contraceptive effect in active immunization studies and, furthermore, the contraceptive effects are reversible in both males and females (Primakoff *et al.*1988).

2.4.1 ZP3 Antigen for Immunological Contraception

In the past two decades various candidate antigens have been extensively studied for immunologic contraceptives. These include spermatozoa antigens (Naz *et al.*1991b; Aitken *et al.*1987) hormonal antigens (Stevens *et al.*1990; Talwar *et al.*1988) and zona pellucida antigens (Yurewicz *et al.*1986; Dean & East, 1986; Isojima *et al.*1986; Sacco *et al.*1986; Paterson & Aitken, 1989; Mahi Brown *et al.*1988; Paterson *et al.*1992). ZP3 emerges as a strong contestant for contraceptive vaccine development for the

following reasons, 1) it possesses sperm receptor activity, 2) the antigens of which it is comprised are not expressed in any other tissue, 3) small amounts of antibodies are enough to block spermatozoa binding to single ovulated oocytes, 4) it is highly immunogenic in most of the animals studied so far 5) it prevents rather than terminates pregnancy, and 6) it can be purified by chromatographic methods.

Porter (1965) showed that when female guinea pigs were immunized with guinea pig ovaries, inhibition of ovulation resulted. Later Shahani *et al* (1969) prepared a rabbit antibody against mouse ovarian tissue and reported that this antibody greatly inhibited fertility *in vivo*. Gonad specific antigens localizing to the sperm acrosome and zona pellucida have been demonstrated by a fluorescent antibody technique with rabbit anti-guinea-pig ovary and anti-guinea pig testis antisera (Porter *et al.*1970). Rabbit antibody against golden hamster ovary, when absorbed with hamster small intestine and lung and then allowed to react in Oucterlony plates produced one precipitin band against hamster ovary and no other bands against eleven other hamster tissues tested (Ownby & Shivers, 1972). Sacco and Shivers (1973a) studied the ovarian specific antigens by an indirect fluorescent antibody technique and localized two major ovarian antigens in the zona pellucida, theca interna and atretic follicles.

The notion of using zona pellucida as the target for a contraceptive vaccine was first put forward by Shivers *et al* (1972) two decades ago. These workers demonstrated the inhibition of sperm-egg interaction by an anti-ovarian antibody that specifically targeted the zona pellucida. Sperm attachment was prevented due to the formation of a precipitate on the outer surface of the zona pellucida. A number of laboratories from different parts of the world have subsequently confirmed the powerful immunogenecity and contraceptive potential of antibodies generated against the zona pellucida. *In vitro* isolated oocytes treated with antibodies against ovarian

extract inhibited sperm attachment to and penetration through the zona pellucida (Ownby & Shivers, 1972; Sacco & Shivers, 1973b; Metchnikoff, 1899). Similarly, antibodies against ovarian extract blocked the fertilization of golden hamster eggs *in vitro* by binding to the zona pellucida and preventing sperm attachment and the same antibody also rendered the animal sterile for 12 days when given as an intraperitoneal injection (Oikawa & Yanagimachi, 1975). Rabbit antibodies produced against hamster ovarian antigens blocked fertilization *in vitro* and *in vivo* and the formation of a clear precipitate around the zona pellucida was responsible for this effect. The specificity of the antizona antibodies used in such experiments was indicated when radiolabelled antibody against hamster ovarian antigens was given *in vivo* was found localized in the ovary to a higher degree than in any other tissue tested (Yanagimachi *et al.*1976).

2.4.2 Active Immunization

Practically all the initial immunization studies were done with crude ovarian extracts. Heterologous, heat-solubilized hamster zonae pellucidae was employed for the first time in active immunization studies by Gwatkin et al (1977) and the infertility induced was found to be reversible and without any side effects. However, the ova recovered from these immunized animals were found to have lost their shape and were fragile. Anti-rat ovary antisera prepared in rabbits significantly inhibited fertilization of rat eggs *in vitro* but also inhibited fertilization in the mouse and hamster indicating cross reactivity between the zona antigens expressed by these respective species (Tsunoda & Chang, 1976b). The observations of cross reactivity between rat and mouse zonae pellucidae by indirect immunofluorescence, complement fixation and immunoprecipitation tests facilitated the use of heterologous ZP in active immunization experiments involving the rat and mouse (Aitken & Richardson, 1980).

Rats were actively immunized with mouse ovarian antigen, and antibody titres were monitored by immunofluorescence, immunoprecipitation and spermatozoa binding assays. The antibody titre correlated with the onset and duration of infertility in the rats during the course of the study period. Most of the animals remained infertile without any side effects (Aitken & Richardson, 1981d). Further studies demonstrated that rabbit anti-hamster ovarian antibody could block sperm-egg binding in a homologous systems and that the antibody was effective when passively administered *in vivo* without affecting the oestrous cycle and ovulation. In addition, anti-hamster ovarian antibody when added to embryos at different stages (2, 4, 8, 16 cell and morula stage) *in vitro* failed to implant when subsequently transferred to the uterus, suggesting that the antibodies prevented zona shedding before implantation (Dudkiewicz *et al.*1975). Similar findings in the mouse have been reported using rabbit anti-mouse ZP antibodies (Tsunoda & Chang, 1978). The cross linking induced by the anti-ovarian antibodies rendered the ZP resistant to trypsin and pronase, both of which are normally able to dissolve the zona pellucida without difficulty. Canine ovarian antibodies also prevented *in vitro* fertilization of canine oocytes and the implication of these findings for canine birth control has been discussed (Mahi & Yanagimachi, 1979). Rats immunized with cross reactive mouse zonae pellucidae showed a complete block of fertility without any side effects. After a decline in antibody titres there was an uncomplicated return of fertility (Aitken & Richardson, 1981a). It has been suggested that the anti-zona antibodies can inhibit fertility even after fertilization, in view of evidence produced by post mortem examination of immunized animals, demonstrating the presence of fertilised ova surrounded by fragile, fractured zonae (Aitken & Richardson, 1981a). In subsequent studies, the duration of infertility following active immunization with heterologous pure ZP was correlated with antibody titres measured by complement fixation, immunofluorescence

and immunoprecipitation (Aitken & Richardson, 1981c). Similarly bitches immunized with isolated porcine zonae pellucidae developed high antibody titres and expressed prolonged infertility. In contrast, an animal immunized with homologous canine zonae and one control immunised with adjuvant alone did become pregnant. The bitches immunized with porcine zonae did however experience somewhat abnormal cycles although the reason for this was not clear (Mahi Brown *et al.*1982).

The development of large scale isolation methods has enabled the production of useful quantities of zonae from various species such as the pig, (Dunbar *et al.*1978; Dunbar *et al.*1980) rabbit (Dunbar, 1980) and cow (Gwatkin *et al.*1980) for production of antisera and biochemical characterization. Mice immunized with hamster zonae (Gwatkin *et al.*1977) and rabbits immunized with bovine (Gwatkin & Williams, 1978), porcine (Gwatkin *et al.*1980; Wood *et al.*1981), or rabbit zonae (Wood *et al.*1981) had significantly reduced fertility. Immunization of rabbits with intact or heat-solubilized porcine zonae pellucidae completely prevented term pregnancies. No eggs were found after ovulation in the former group while ovulation had not occurred in rabbits immunized with heat-treated ZP (Wood *et al.*1981). Detergent-dissociated porcine zonae or intact rabbit ZP used as immunogen produced variable reductions in fertility, ranging from 30-70%. The antibody titres measured by radioimmunoassay were maximal after the first booster and remained high throughout the experiment (Skinner *et al.*1984). Additional experiments involving the active immunization of rabbits with heat-solubilized porcine ZP revealed that the rabbits had abnormal follicular development, did not ovulate in response to hCG and had elevated serum concentrations of gonadotrophins suggesting disruption of negative feedback control by steroids of ovarian origin. Histological examination of the follicles showed only a few growing follicles after 23 weeks of immunization and the follicles did not contain oocytes (Skinner *et al.*1984).

The cross-reactivity exhibited between primate and porcine zonae pellucidae (Sacco *et al.*1981; Sacco *et al.*1983) has also led to the use of primate models in different active immunization studies. For example studies that have been carried out in cynomolgus monkeys using heat-solubilized porcine ZP (Gulyas *et al.*1983) in 1% Alhydrogel, with monthly boosters. Six of the twelve monkeys became pregnant between six to ten weeks after the first immunization, whereas the remaining monkeys did not conceive, despite regular matings until their antibody titres fell after boosters were stopped. The cycles of five monkeys were irregular and histological examination of the ovaries showed some with developing follicles and some without. The authors suggested that the impurity of the zona preparation may have caused the impairment of ovarian function since repeated injections of contaminating proteins from eggs or follicle cells could raise antisera to those structures. Active immunization studies involving squirrel monkeys using purified porcine zona antigen (PPZA) of 60 kD, which was shown to be free of the other two major glycoprotein families contained in ZP, produced significant antibody titres. Laparoscopic examination of the monkeys revealed that the ovaries of the immunized monkeys were smaller and had fewer follicles than those of control animals. Only two oocytes could be recovered after hCG injection to induce ovulation. The oocytes had evidence of antibodies bound to the ZP suggesting that antibodies can reach the follicles *in situ* (Sacco *et al.*1983).

Studies in bonnet monkeys immunized with 200μg of intact pig zonae pellucidae in Freund's adjuvant indicated that both progesterone and oestradiol concentrations fell following the generation of high anti-zona antibody titres, but normal cyclicity returned when the antibody titres fell to basal level (Bamezai *et al.*1986). A more elaborate study was done using 50 squirrel monkeys with a purified porcine zona pellucida antigen possessing sperm receptor activity (Sacco *et al.*1984). After active immunization, high antibody titres were induced in the monkeys for as

long as 18 months, and the animals experienced infertility. Disturbances in hormonal status along with histological changes were observed in these animals suggesting interference with folliculogenesis, although the initial disturbance was followed by a recovery back to normal ovarian function, as indicated by laparoscopic and hormonal data, despite the presence of high titres of anti-zona antibodies (Sacco *et al.*1987). Dunbar *et al.*(1989) have used muramyl dipeptide, a synthetic adjuvant, together with purified porcine pig zona pellucida antigen (ZP1) to immunize baboons. These animals showed decreased levels of oestrogen throughout the menstrual cycle, and two of the animals ceased ovulation. Furthermore ovarian sections from these animals revealed changes in the number and normality of ovarian follicles.

The ovarian pathology evident from the active immunization experiments involving primates and rabbits has also been observed in bitches actively immunized with crude or partially purified porcine zonae pellucidae. In such cases an elevation of oestradiol levels and a reduction in plasma progesterone concentrations was observed (Skinner *et al.*1984; Sacco *et al.*1991; Paterson *et al.*1992). However when purified porcine zonae pellucidae was used as an immunogen there was an inhibition of fertility without loss of ovarian function, in all animals except one. All three different adjuvants (Freund's, CP 20-961, and alum) produced antibody titres sufficient to suppress fertility (Mahi Brown *et al.*1985). Other reports indicated no observable adverse effects following active immunization (Shivers *et al.*1981). However, recent studies in the marmoset have provided further evidence of ovarian dysfunction even in animals that have ben actively immunized with highly purified porcine ZP3 antigens.

Active immunization studies employing of porcine ZP3 preparations such as the deglycosylated polypeptide core of Mr=32,000 (DGZP-32) and the fully glycosylated 55,000 (ZP3) have been used to actively immunize marmoset monkeys in order to investigate the contraceptive efficacy and safety of such preparation (Paterson

et al. 1992). The DGZP-32 with non-ulcerative Freund's adjuvant was not able to generate antibody titres of the same magnitude as glycosylated ZP3. However, despite the difference in immunogenicity between the native glycoprotein and the core DGZP-32, both antigen sources exhibited contraceptive potential *in vivo*. Nevertheless, during the course of these active immunization studies, animals exhibited abnormal ovarian cyclicity. Ovarian histopathology in the high antibody titre animals was associated with severe follicular atrophy, absence of primordial follicles and the presence of a large number of follicular clusters with no evidence of active folliculogenesis. When the immunogen consists of deglycosylated peptide, the onset of this pathology was delayed and took about 2 years to manifest itself. Moreover, when the cessation of ovarian cyclicity did become apparent, the pathology did not reverse as antibody titres decline; it was permanent. It is important to note that there was a considerable lag time before the onset of these pathological changes (Paterson *et al.*1992).

Progress towards the development of anti-zona vaccines that are free from detrimental effects on the ovary has been made using the mouse as a model. A mouse monoclonal antibody known to block fertilization in mice after passive immunization was used to screen a cDNA encoding ZP3, which was randomly cleaved to 200-1000 base pair fragments. A synthetic peptide was synthesized based on the sequence derived from the epitope library screening. The synthetic peptide, containing a B cell epitope, was conjugated to a macromolecular carrier protein known to provide helper T cell epitopes. This study demonstrated that repeated immunization of female mice with a mouse ZP3 peptide-KLH conjugate can induce long term infertility without any ovarian histopathology. It was suggested that the absence of ovarian histopathology was due to a lack of ZP3 T cell epitopes which could otherwise have a cytotoxic effect on the primordial follicles (Millar *et al.*1989).

2.4.3 *In Vitro* Studies

Anti-ovarian antibodies have been shown to block fertilization *in vitro* in a number of mammalian species tested so far (Shahani *et al.*1969; Porter, 1965; Shivers *et al.*1972; Tsunoda & Chang, 1976b). The inhibition of sperm attachment to the zona pellucida is achieved through the occlusion of sperm binding sites on the zona surface by the cross-linking activities of the anti-ZP antibodies, which result in the formation of a precipitate on the ZP surface (Aitken *et al.*1981; Aitken & Richardson, 1981b). However, when the cross-linking activity of such antibodies is destroyed through the preparation of Fab fragments, neither immunoprecipitate formation nor the inhibition of sperm-zona interactions was observed (Aitken *et al.*1982; Ahuja & Tzartos, 1981). Monoclonal antibodies specific for either ZP-2 or ZP-3 also completely block *in vitro* fertilization at relatively low concentrations ranging from 0.4 to 75 micrograms/ml. The contraceptive effect requires the presence of the zona and appears to involve the inhibition of zona penetration by the spermatozoa rather than the occlusion of sperm binding sites (East *et al.*1985). Monoclonal antibodies have also been described that, while directed against single epitopes, can block sperm binding to the ZP even though there is no immunoprecipitate formation (Isojima *et al.*1984; Koyama *et al.*1985; East *et al.*1985). In such cases, the antibody is not being effective through steric hindrance mechanism but must be targeting on antigenic determinant in close proximity to the binding site itself. Bamezai *et al.* (1988) have identified a mouse monoclonal antibody against porcine ZP that inhibits sperm penetration in dogs. Some of the monoclonal antibodies developed against porcine and rabbit ZP fail to block sperm binding, although they react with the antigenic determinants present on the ZP (Drell & Dunbar, 1984). Such antibodies must presumably be targeting antigenic determinants distant from the sperm binding site. In general, most of the monoclonal antibodies

generated against the ZP are not effective in disrupting fertilization by blocking sperm-zona interaction, either at the level of sperm binding to the zona surface or by preventing penetration of the zona matrix, even though immunological cross-reactivity may be demonstrable (Isojima *et al.*1984; Koyama *et al.*1985). The addition of a second, polyclonal antiserum directed against mouse immunoglobulin can generate bioactivity by facilitating immunoprecipitate formation and masking the sperm receptor sites by steric hindrance (Koyama *et al.*1985). An important exception, in terms of this need for a second cross linking antibody is the work of Mori et al (1985) who generated two monoclonal antibodies; one of them formed an immunoprecipitate during immunoelectrophoresis while the other did not. However, both antibodies block human sperm-zona interactions without a need for a second, cross-linking antibody (Mori *et al.*1985). Such results emphasise that immunoprecipitation formation is not an absolute requirement of the contraceptive action of antibodies directed.

Recently, a different ZP antigen, identified in the porcine ZP and termed ZP4, was purified by SDS-PAGE, and used to raise monoclonal antibodies. Five of these monoclonal antibodies reacted with intact ZP of porcine, human and rabbit oocytes and all five of them showed a strong blocking effect on the human spermatozoa-zona interaction without the need for a second antibody. These antibodies appeared to recognize a key epitope on the ZP, although it is not known whether their inhibiting activity might have been due to steric hindrance of the sperm receptor through the formation of immune complexes or due to direct interference with the receptor site itself. The investigators have observed that the monoclonal antibody to ZP4 does not block the initial spermatozoa binding to human ZP; instead, most of the spermatozoa attached to ZP gradually dissociated within a few hours of the subsequent incubation period. As a result, the number of spermatozoa bound and penetrating the ZP at the

final stage of the incubation was markedly reduced (Koyama *et al.*1991). It is therefore possible that antibodies directed against ZP4 are effective in suppressing the secondary binding of acrosome-reacted spermatozoa to the ZP, rather than the disruption of the primary recognition event.

2.4.4 Passive Immunization Studies

Antisera against ovarian antigens could induce infertility in a variety of species such as hamster (Oikawa & Yanagimachi, 1975), mice (Jivek & Pavlok, 1975), and rats (Tsunoda & Chang, 1976b) by passive immunization. Fertilisation of golden hamster eggs was blocked both *in vitro* and *in vivo* by antibodies produced in rabbits against specific hamster ovarian antigens. Anti-hamster ovarian antigen antibody, when injected into animals, bound to the ZP and blocked fertility for four oestrous cycles and thereafter fertility returned to normal levels. These results suggested that the antibodies did not cause destruction of the female reproductive organs. Passive immunization with radiolabelled anti-hamster ovarian antigen antibodies indicated that the antibodies were concentrated in the ovary during the period of infertility (Yanagimachi *et al.*1976). A single intraperitoneal injection of rabbit antisera against denuded mouse eggs and isolated ZP inhibited fertilization in mice for 25-30 days. After fertilization the antibodies did not affect the embryonic development of foetuses (Tsunoda & Chang, 1978).

A single passive immunization of anti-zona antibodies in mice was found to provide protection against conception for 24-30 days (Sacco, 1979). Dean and East (1986) have demonstrated that passive immunization of mice with antibodies specific for either ZP-2 or ZP-3 inhibited fertilization *in vivo* and *in vitro*. This effect was observed with ng/ml quantities of antibody. It appeared that the antibodies did not preclude sperm binding but rather prevented sperm penetration of the zona through

their cross linking activity. The contraceptive effect of passive immunization is fully reversible and this reversibility is associated with a loss of antibody binding to the zona pellucida. Antibodies to ZP-2 or ZP-3 had no other adverse effects on either *in vivo* or *in vitro* pre-implantation development (Dean & East, 1986). The prolonged periods of infertility observed following the induction of passive immunity appear to be due to the ability of these antibodies to bind to the zonae pellucidae of follicular as well as ovulated oocytes. As a result, the ova liberated from the ovary over six or seven successive cycles will have been affected by the anti-zona antibodies. Ova recovered from passively immunized animals possess an immunoprecipitate on the outer surface of the zona pellucida and an increased resistance to solublization (Tsunoda & Chang, 1976b; Tsunoda & Chang, 1976a; Tsunoda & Chang, 1978). Most interestingly, monoclonal antibodies developed against ZP2, which is the secondary sperm receptor, are effective in blocking the formation of 2-cell embryos (East *et al.*1984b). Thus, antibody binding to the proposed primary sperm receptor (ZP3) does not appear necessary to prevent fertilization.

2.4.5 Ovarian Pathogenicity

Experimental results obtained to date reveal that after active immunization of female rabbits or primates with porcine ZP glycoprotein, there is an interruption of follicular development, which coincides with the stage at which the ZP first appears as an extracellular matrix between the oocyte and follicular cells. In actively immunized rabbits there was a noticeable reduction in ovarian size and weight as early as 7 weeks after primary immunization. Progesterone secretion in response to hCG was completely absent within 20 weeks of immunization and FSH and LH secretion was strikingly increased over control values. The alteration in ovarian function was associated with the presence of serum antibodies to the three major porcine ZP

glycoproteins. Histopathological analysis demonstrated that there was an increase in the number of follicles with atretic oocytes and the complete disappearance of growing follicles as well as corpora lutea within 30 weeks post immunization. The number of primordial follicles present in the long-term (40-48 weeks) immunized animals was reduced. This decrease was accompanied by an increase in the number of oocyte-free follicular cell clusters. This whole effect appeared to be due to a humoral immune response rather than a cell-mediated response, since there was no sign of macrophage or leucocyte infiltration or inflammation in the ovaries even at early time points (e.g. 2-21 days) after the primary immunization (Skinner *et al.*1984).

Immunisation with crude porcine ZP caused abnormal oestrous cycles and infertility in bitches with high antibody titres against crude porcine ZP. In such animals histological examination and steroid hormone profiles strongly supported a failure of ovulation. In contrast bitches with low antibody titres against crude porcine ZP seemed to have ovulated, as indicated by elevated serum progesterone levels. The bitches immunized with purified porcine ZP had moderate titres and their cycles were normal. Thus, in general, purified porcine ZP preparations did not affect the steroid profile of bitches during their first cycle after immunization, but by the time of the second cycle one animal which had received 6 injections, exhibited an abnormal cycle associated with an abnormal steroid profile, similar to that in the bitches exhibiting high antibody titres following immunization with crude porcine ZP material (Mahi Brown *et al.*1985). In some actively immunized bitches, oocytes did not ovulate or might have ovulated in a degenerated condition. In some bitches, ovulation did not occur because of a failure of follicles to survive beyond the primordial stage. In bitches immunised with ZP, the most common histological finding in the ovaries was follicular cysts and, in some cases, loss of oocytes from the follicles. It was suggested that the condition observed seemed to reflect premature ovarian failure in women. It

has been postulated that the loss of oocytes in actively immunized animals might result from the interruption of communication between oocytes and the granulosa cells because of interference with the cytoplasmic processes that pass from the corona cells through the ZP, as a result of anti-zona antibody precipitation on the ZP surface (Mahi Brown *et al.*1988).

Bamezai *et al* (1986) have reported that bonnet monkeys immunized with porcine ZP were infertile. Steroid secretion and ovarian cycles were disturbed during the period of high anti-zona antibody activity, but returned to normal once antibody levels had dropped. A reduction in the development of ovarian follicles and a decrease in oocyte survival, along with reduced levels of oestrogen and variable levels of progesterone secretion were observed in squirrel monkeys immunized with purified porcine ZP3. After 10 months of immunization, there appeared to be a recovery in the ovarian steroid response, despite the presence of high antibody titres, but fewer oocytes were recovered from ZP3 injected monkeys (Sacco *et al.*1987).

On the other hand, active immunization of bonnet monkeys with purified ZP3 showed adverse effects on the ovary depending upon the type of adjuvant used. Animals immunized with complete Freund's adjuvant showed profound ovarian follicular atrophy, and an absence of Graafian follicles and corpora lutea in the ovaries. The secondary follicles were reduced in size and appeared as cellular aggregates bound by a continual basal lamina but the primordial follicles continued to survive. No leucocytic infiltration was noticed at any stage of follicular atrophy or resorption. Bonnet monkeys immunised with ZP3 using sodium phthalyated lipopolysaccharide as adjuvant did not show any noticeable histological changes in the ovaries. The primordial follicles were abundant and present all along the periphery of the ovarian cortex. The secondary follicles and large antral follicles displayed normal morphology and signs of ovulation as indicated by the presence of corpora lutea

(Upadhyay *et al.*1989). It has been suggested that mycobacterial antigens present in CFA could trigger autoimmunity against auto-antigens or might activate cytotoxic T cells that could possibly react with certain ovarian antigens (Shoenfeld & Isenberg, 1988). Similar observations have also been made in control animals receiving CFA alone which showed altered cyclic ovarian activities (Aitken *et al.*1984; Sacco *et al.*1987; Sacco *et al.*1986), whereas injection of pertussis toxin as an additional adjuvant did not significantly alter the severity or prevalence of ovarian disease (Rhim *et al.*1992). In a further study, a synthetic mouse ZP peptide containing the B cell but not T-cell epitope coupled to a carrier protein produced long-lasting contraception without any ovarian histopathology (Millar *et al.*1989).

The hormonal impact of immunization with solubilized ZP and ZP3 has been recorded in rabbits. Both native ZP3ß and ZP3ß/endo-ß-galactosidase-digested (EBGD) immunogen produced elevated levels of LH and FSH and loss of hCG-induced progesterone secretion. Whereas the rabbits immunised with ZP3å/EBGD, ZPå/DG and ZP3ß/DG showed no significant elevations of gonadotrophins and continued to display cyclic progesterone secretion in response to hCG (Keenan *et al.*1991). Recently, a study was undertaken to analyse the importance of the carbohydrate moiety in the aetiology of impaired ovarian function by comparing the consequences of inducing immunity in the marmoset against porcine ZP3 and a peptide derived from this molecule, DGZP32, the chemically deglycosylated core of ZP3ß. Regardless of the nature of ZP antigens used for the induction of immunity, all animals showed signs of profound follicular atrophy. The ovaries appeared small with no primordial follicles and contained a large number of follicular clusters with no evidence of folliculogenesis. The plasma progesterone profiles indicated no initial disturbance to the ovaries, but the presence of antibody for a prolonged period of time (>18 months) led to a complete fall in progesterone levels. Such results suggested that

it is not only the oligosaccharide side chains that might bear the epitopes responsible for the induction of pathological changes in the ovaries of actively immunized animals (Paterson *et al.*1992).

In another study, immunization of mice with rat ZP had a significant effect on small antral follicles suggesting that the pre-antral stage was most sensitive to the effects of immunization. The percentage of all large follicles (pre-antral to large antral) with visible oocytes was significantly less in the immunized mice (Mahi Brown *et al.*1992). Furthermore, this study demonstrated that a significant cellular response can be generated by measuring the proliferation of T cells in response to ZP *in vitro*, although this study was not extended to determine whether the ovarian pathogenicity was due to a cellular immune response to ZP (Mahi Brown *et al.*1992). The importance of cellular immune responses in the aetiology of ovarian pathology was emphasised by an experiment using a synthetic 15 amino acid mouse ZP3 peptide 328-342, which could elicit oophoritis in B6AF1, BALBc/By, and A/J mice. This synthetic peptide contained both B and T cell epitopes, suggesting that both epitopes might be involved in autoimmune oophoritis in mice. However, adoptive transfer of the disease by lymph node cells and two $CD4^+$ T cell lines derived from mice immunized with peptides ZP3 330-342 and ZP3 328-340 strongly suggested that T cells were involved in ovarian atrophy. This study also further demonstrated in actively immunised mice the presence of infiltrating inflammatory cells as early as day 6 post immunization with a maximum effect on day 21 (Rhim *et al.*1992). Additional experiments have been carried out in rabbits with different forms of ZP preparations like solubilized porcine ZP (SIZP), purified porcine ZP3å and ZP3ß, purified endo-ß-galactosidase (EBGD) digested glycoproteins ZP3å-(EBGD), ZP3ß-(EBGD)(each about 30% deglycosylated), chemically deglycosylated (DG) core proteins ZP3å-DG and ZP3ß-DG. Rabbits immunised with SIZP and ZP3 showed greatest follicular

growth disruption as early as 6 and 12 weeks after initial immunization. These animals showed significantly fewer primary, secondary and tertiary follicles up to 36 weeks of this study (Keenan *et al.*1991). Histopathological examination of ovaries from ZP3ß-EBGD-immunized rabbits showed a reduction in all stages of follicle development, whereas ZP3ß-DG showed a significant decrease in primary follicles after 18 weeks of immunization but no significant follicular reduction of secondary and tertiary follicles. On the contrary, rabbits immunised with ZP3å-EBGD and ZP3å-DG showed normal follicular development of all stages with normal ovarian morphology (Jones *et al.*1992a). When a different antigen (porcine ZP4) was used as a immunogen, there was a milder degenerative change in hamster ovarian sections, which returned to normality during the subsequent decline of antibody titres (Hasegawa *et al.*1992).

2.5 Summary

The molecules of which the zona pellucida is comprised are thought to play many important roles during the process of fertilization. Molecular cloning of the sperm receptor has helped to understand the structure and function of ZP3. Also recombinant technology has helped to produce abundant quantities of this material for further research on contraceptive vaccine development, including the mechanism by which the anti-fertility effects are achieved and the pathophysiology of ovarian dysfunction.

3 Cloning and Sequencing of Marmoset ZP3 Gene

3.1 Introduction

The discovery of the double helix structure of DNA by Watson and Crick (1953) was a significant breakthrough in molecular biology. In the 1970s, the advent of techniques for the *in vitro* manipulation of DNA like subcloning and sequencing, completely changed our understanding of the biology of living organisms. These new techniques allowed the introduction of manipulated genetic material into an organism in such a way as to replicate and be passed on to the progeny (Gingold, 1988). In order for the foreign gene to replicate, it must be attached to a molecule that is capable of replicating in a bacterial cell. A bacterial species such as *E. coli* will not recognize a foreign gene; so it is essential to clone the foreign DNA into a carrier molecule that is capable of integrating into the bacterium and replicating. Carrier molecules composed of extrachromosomal DNA, termed plasmids, found in the bacterial species serve this purpose (Broda, 1979). To clone a foreign gene into a vector the DNA has to be cut at a particular site in order to ligate it into the vector sequence. It was found that bacteria could cut up phage DNA on entry into the cell and the properties of these cutting enzymes, called restriction enzymes or endonucleases, provided an invaluable tool for cutting DNA at defined sites (Dussoix & Arber, 1962). The enzyme DNA ligase helps to form covalent bonds between the cut ends of the DNA.

Many of the problems associated with protein sequencing have been solved by DNA sequencing procedures (Sanger *et al.*1977; Maxmam & Gilbert, 1977). The chain termination technique using dideoxynucleotides has proven extremely valuable

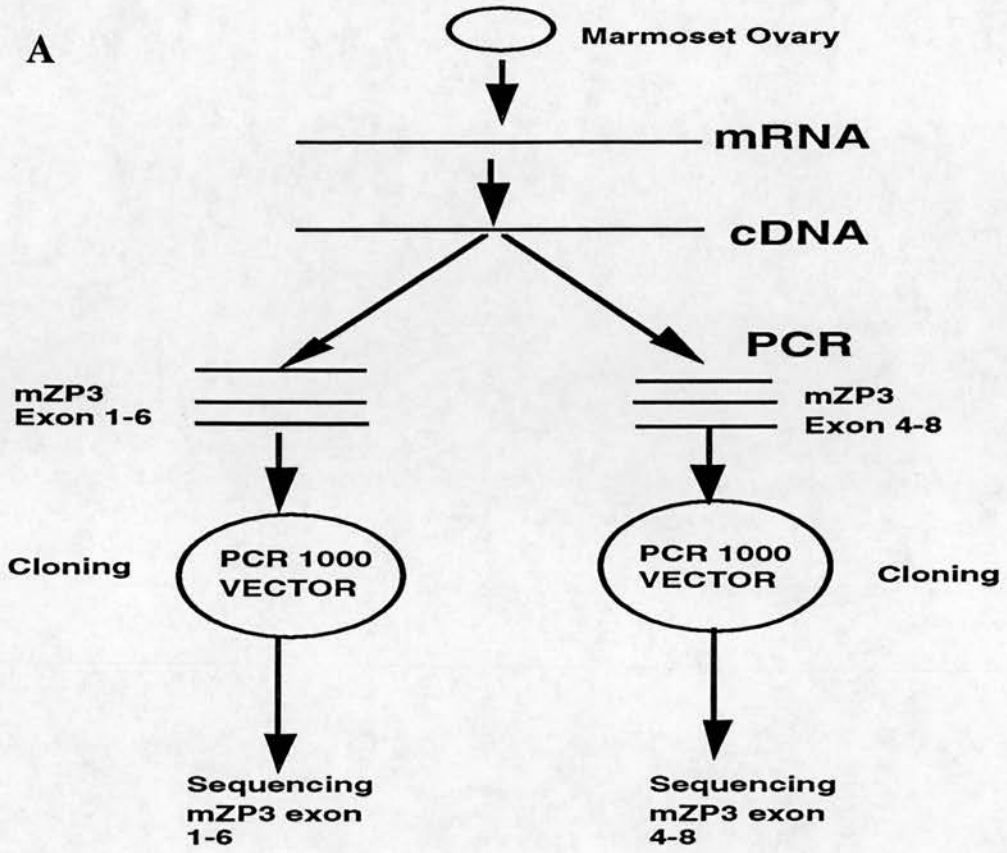
for rapid DNA sequencing. The polymerase chain reaction (PCR) has also speeded up many molecular biology procedures like amplification of DNA from a single DNA molecule, rapid sequencing, amplification of small amounts of mRNA by reverse PCR or cDNA amplification, and cloning. The purpose of this section of the research was to use such molecular biology techniques to clone and sequence the marmoset ZP3 gene and thence determine the primary amino acid structure of this molecule.

The importance of ZP3 stems from the role this proteins play during fertilization: the binding of spermatozoa to the egg, the induction of the acrosome reaction and the prevention of polyspermy. All earlier studies on the structure and function of ZP3 utilised material manually isolated from the ovaries of a particular species. Under such circumstances, it was rather difficult to obtain sufficient material to determine the primary amino acid sequence of ZP3 or the structure of the gene encoding this protein. This is particularly true of the ZP from primates, including man, which are extremely difficult to obtain in appreciable quantities. As a consequence, molecular biology techniques have been used to clone and sequence the genes encoding for the ZP3 protein in a variety of different mammalian species. Thus, the primary amino acid structure of human, mouse and hamster ZP3 have been elucidated through gene cloning and sequence analysis (Kinloch *et al.*1988; Kinloch *et al.*1990; Ringuette *et al.*1988; Chamberlin & Dean, 1990). The results indicate that ZP3 has no homology with other proteins; it is a sex-specific gene that is expressed uniquely in growing oocytes during active folliculogenesis and is apparently undetectable in the primordial follicle population. Comparison of the mouse and hamster ZP3 proteins has revealed an 81% sequence homology, whereas the similarity between these amino acid sequences and human ZP3 is around 60% (Figure 3.8). The polypeptide core of all three proteins is approximately 420 amino acid residues in length, in which two potential N-linked glycosylation sites are conserved. Additional

N-linked carbohydrate addition sites and numerous positions for O-linked sugars probably provide the protein with the structural information that is essential for the species specificity of sperm-zona interaction. Thus, despite the conserved primary amino acid structures of the polypeptide core, differential glycosylation leads to considerable heterogeneity in the molecular mass of the secreted product giving an apparent M_r of 83kD for the mouse ZP3 and a spectrum of charge isomers ranging from 50 to 60 kD for human and hamster ZP3 (Moller *et al.* 1990; Shabanowitz, 1990).

Conservation and elucidation of the primary structure of human ZP3 have been particularly important in the context of contraceptive vaccine development. Antibodies against the deglycosylated peptide core of porcine ZP3 have been shown to block the fertilization of human ova *in vitro* and, moreover, to disrupt the fertility of actively immunised marmosets *in vivo* (Paterson *et al.* 1992). Such active immunization experiments have been particularly informative since they have revealed important side effects of anti-ZP3 immunity, involving the depletion of the primordial follicle population and the onset of a premature menopause. While the induction of immunity against heterologous zona antigens has generated interesting information, such studies may not accurately reflect the consequences of developing immunity against a homologous peptide.

The ideal protocol for the evaluation of a contraceptive vaccine would be one in which an animal model, such as the marmoset monkey, was immunized with homologous ZP3 peptides that exhibited perfect sequence homology with human ZP3. In order to develop such a model the primary amino acid structure of marmoset ZP3 would have to be elucidated and compared with its human counterpart. The present study was designed to achieve this objective.



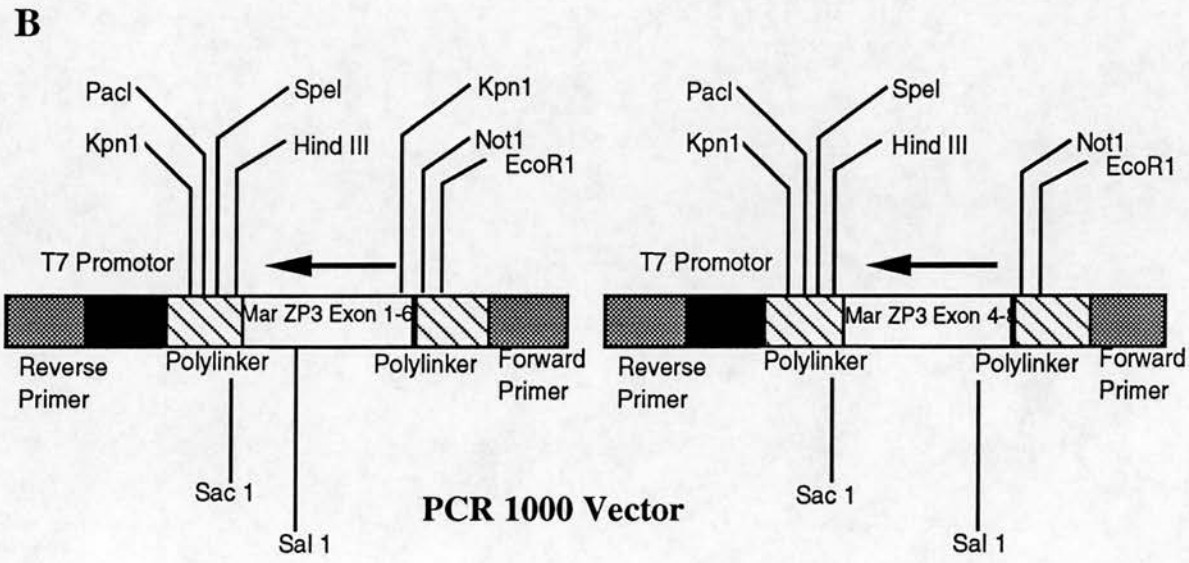


Figure 3.1 Schematic diagram of the marmoset ZP3 cloning and sequencing procedure (A). PCR 1000 Vector employed for cloning the mar ZP3 cDNAs (B).

3 2 Materials and Methods

3.2.1 Poly(A)-mRNA Isolation

Marmoset ovarian material was collected from the Medical Research Council, Primate Colony, Edinburgh for the purpose of mRNA isolation. The ovarian tissue was frozen in liquid nitrogen and transported to the laboratory and stored at -80°C until use where the tissue was homogenised using a glass hand-homogenizer (Kontes Biotechnology, Vineland, NJ, USA). For the mRNA isolation, Dynabeads, mRNA purification kit (Dynal (UK) Ltd, New Ferry, Wirral, UK) was used. 1ml lysis/binding buffer [100 mM Tris-HCl, pH 8.0; 500 mM LiCl; 10 mM EDTA, pH 8.0; 1% lithium dodecylsulphate (LiDS); 5 mM dithiothreitol (DTT)] was added to the homogenizer and homogenised manually for 1-2 min. The lysate was transferred to a microfuge tube and spun for 30 seconds. The supernatant was mixed with 1.5 mg of Dynabeads Oligo (dt) in 100 µl lysis/binding buffer and allowed to anneal for 3-5 minutes on ice. The tube was placed in the Dynal MPC-E-1 magnetic stand and the supernatant removed. The bound oligo (dt) was washed 3 times with 0.5 ml washing buffer (10 mM Tris-HCl, pH 7.5; 0.15 M lithium chloride; 1mM EDTA; 0.1-0.3% SDS) using the Dynal MPC-E-1 separator. The bound Poly (A)-mRNA was eluted with 20µl of elution buffer (2 mM EDTA, pH 7.5) by incubating the tube in a 65°C water bath for 2 minutes.

3.2.2 Marmoset Ovarian cDNA Synthesis

The Poly (A)+RNA of marmoset ovary was used to synthesize the cDNA by using Amersham's cDNA synthesis system plus (Amersham, UK). The first strand cDNA synthesis was made as follows, 4µl of 5x 1st strand synthesis reaction buffer, 1µl of sodium pyrophosphate solution, 2µl of human placental ribonuclease inhibitor,

deoxynucleoside triphosphate mix, 1µl of Oligo dt, 2µl of marmoset ovarian poly (A)+ RNA (2 µg), 9µl of water and reverse transcriptase, 1.5µl containing 40 units of activity and then incubated at 42°C for 40 minutes.

3.2.3 Second Strand cDNA Synthesis

To the above first strand cDNA mixture, was added 37.5µl of second strand synthesis reaction buffer, 1µl (0.8 units) of *E.coli* ribonuclease H, 6µl (23 units) of *E.coli* DNA polymerase 1, and 34µl of water. The above reaction was carried out at 12°C for 60 minutes; 22°C for 60 minutes and 70°C for 10 minutes. After a brief spun, 2µl containing 2 units of T4 DNA polymerase was added and incubated at 37°C for 10 minutes. The reaction was stopped by adding 2µl of 0.5M EDTA pH 8.0. The cDNA reaction mixture was extracted using 1 volume of 1:1 phenol chloroform (1 part TE buffer-saturated phenol, pH 7.8; 1 part chloroform). The solution was vortexed for 1 minute and then microcentrifuged at 12 000 x g for 5 minutes. The upper aqueous phase was transferred to a new tube and an equal volume of chloroform added. After vortexing for 1 minute, this was spun at 12 000 x g for 5 minutes. The upper aqueous phase was again transferred to a new tube and an equal volume of 4M ammonium acetate was added together with twice the combined volume of cold (-20°C) ethanol which had been cooled at -20°C for a minimum of 30 minutes. After centrifuging at 12 000 x g for 5 minutes, the supernatant was carefully removed and the pellet resuspended in 1 ml of 70% ethanol. This was spun again, the supernatant carefully aspirated and the cDNA pellet dried under vacuum. The pellet was resuspended in 10µl of TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0).

3.2.4 Primer Synthesis

Oligonucleotide primers were made using an Oligonucleotide synthesizer (Applied Biosystems, Warrington, Cheshire, UK). The primers were removed from the column using 2ml ice-cold ammonia and deprotected by incubating overnight in a 55°C water bath. The deprotected primers were ethanol precipitated and stored at -20°C as aliquots.

3.2.5 Agarose Gel Electrophoresis

Agarose gels were run using a Mini submarine agarose gel unit (Model HE33; Hoefer Scientific Instruments, Newcastle, Staffs, UK). 1% agarose gels (SeaKem GTG Agarose; FMC Bioproducts, High Wycombe, Bucks, UK) in 0.5 x TBE containing 0.3µg/ml ethidium bromide (EtBr; Sigma, Poole, Dorset, UK) were run at a constant voltage of 80V for 1-2 hours using 0.5 x TBE as the electrode buffer. Low melting point 2% agarose gels in (Nusieve GTG agarose; FMC Bioproducts) 1 x TAE containing 0.3µg/ml EtBr were run at a constant voltage of 45V for 2 hours at 4°C using 1 x TAE as the electrode buffer.

3.2.6 Polymerase Chain Reaction

The marmoset ovarian cDNA was used as template to amplify marmoset cDNA 1-6 using the polymerase chain reaction (PCR; Saiki et al, 1985, 1988; Mullis & Faloona, 1987). PCR was performed using a GeneAMP PCR Reagent kit with AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) using two primers synthesized as described in Section 3.2.4 and a programmable heating block (Hybaid, Teddington, Middlesex, UK). Marmoset ZP3, exon 1-6 corresponding to human ZP3 (Chamberlin & Dean, 1990) was amplified with oligonucleotide primers 1, 5'-TGCAGGGTACCATGGAGCTATAGGC-3' and primer 2, 5'-

ATCACACCATCGTGGAC-3' in 35 cycles of PCR. The final concentrations of the components in the 100µl reaction mixture were AmpliTaq DNA Polymerase, 2.5 Units; dNTPs each 200µM; 1x reaction buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.5 mM $MgCl_2$, and 0.001% (w/v) gelatin); 0.5µM primer 1, 0.5µM primer 2, The PCR reaction involved 35 cycles of incubation at 94°C for 2 minutes to achieve the initial melt, then 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes followed by a final extension at 72°C for 15 minutes. The PCR reaction products were checked by electrophoresis in a submerged 1% agarose gel in 0.5 x TBE buffer run at a constant voltage of 80V for 1 hour.

Marmoset ZP3, exon 4-8 corresponding to human ZP3 (Chamberlin & Dean, 1990) was amplified with oligonucleotide primers 3, 5'-CAGGTGGCAGGTGATGTA-3' and primer 4, 5'-CTTCTTTTATTCGGAAGCAGACACAGG-3' in 30 cycles of PCR. The final concentrations of the components in the 100µl reaction mixture were AmpliTaq DNA Polymerase, 2.5 Units; dNTPs, each 200µM; 1x reaction buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.5 mM $MgCl_2$, and 0.001% (w/v) gelatin), 0.5µM primer 3, and 0.5µM primer 4. The PCR reaction involved 30 cycles incubation at 94°C for 2 minutes to achieve the initial melt, then 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes followed by a final extension at 72°C for 15 minutes. The PCR reaction products were checked by electrophoresis in a submerged 1% agarose gel in 0.5 x TBE buffer run at a constant voltage of 80V for 1 hour.

3.2.7 Cloning into PCR 1000 Vector

The products of 12 independent PCR runs were pooled and the amplified cDNA fragments of marmoset ZP3 exons 1-6 and 4-8 (0.8 and 0.6kb respectively) so generated were purified by agarose gel electrophoresis using Geneclean II kit (Geneclean II kit, Bio 101 Inc. La Jolla, USA) for cloning into the vector. The marmoset ZP3 exon 1-6 fragment was ligated with PCR 1000 vector (TA cloning kit, Invitrogen, Burford, UK). The ligation mixture contained 2µl of PCR™ vector (25ng/µl), 2µl of marmoset ZP3 exon 1-6 DNA (50ng/µl), 1µl of 10 x ligation buffer, 5µl of water, and 1µl of T4 DNA ligase. Ligation was carried out at 12°C, overnight. The following day, frozen competent *E. coli* cells (INVåF') (TA cloning kit, Invitrogen, Burford, UK) were used for transformation. 1µl of the ligation mixture was added to the competent cells and mixed by tapping gently. The tube was placed on ice for 30 minutes and then incubated for exactly 60 seconds in a 42°C water bath followed by a 2 minute storage on ice. The cells were immediately resuspended in 1ml of prewarmed (37°C) SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) and incubated at 37°C for 1 hour with shaking in 17 x 100mm polypropylene tubes. The cells were then plated on LB plates containing 50µg/ml kanamycin, 40µg/ml X-gal and 0.5mM IPTG (Isopropyl ß-D-thiogalactoside) and grown overnight at 37°C. E. Coli cells transformed with recombinant plasmids resulted in the formation of white colonies. Several white (putative positive) colonies were grown up in LB medium containing 50µg/ml kanamycin, at 37°C, overnight and the plasmids purified using Magic Mini-Prep columns (Promega, Southampton,UK). To identify positive clones, the purified plasmid was restriction map analysed by digesting with Nco1 restriction enzyme (Section 3.2.8), and a band of the appropriate size resolved on a 1% agarose gel before being isolated and subjected to DNA sequencing.

The marmoset ZP3 exon 4-8 PCR fragments were also cloned in the PCR 1000 vector as described in section 3.2.7. To identify positive clones, the purified plasmid was digested with Nco1 and Sac1 restriction enzyme as described in section 3.2.9, identified as a band of the appropriate size of 600 nt resolved on a 1% agarose gel and finally isolated and subjected to DNA sequencing.

3.2.8 Digestion of Plasmid with Nco1

The restriction enzyme Nco1 was purchased from Promega and used with the restriction enzyme buffer supplied with it. The reaction mixture contained plasmid DNA of marZP3 exon 1-6, 25µl (1.5µg), 3µl of restriction enzyme buffer, 2µl (20 units) of restriction enzyme Nco1, and the reaction was carried out at 37°C for 1-2 hours.

3.2.9 Digestion of Plasmid with Nco1 and Sac1

The restriction enzyme Nco1 and Sac1 were purchased from Promega and used in conjunction with the restriction enzyme buffer from Pharmacia 'One-Phor-All'. The reaction mixture contained plasmid DNA of mar ZP3 exon 4-8, 25µl (1.5µg), 4µl of restriction enzyme buffer, 3µl (30 units) of restriction enzyme Nco1, 3µl, (30 units) of restriction enzyme Sac1, 5µl of water, and the reaction was carried out at 37°C for 1-2 hours.

3.2.10 Alkaline Denaturation of DNA

The plasmid DNA was denatured using alkaline treatment. The DNA was denatured in 0.2M NaOH, 0.2mM EDTA for 30 minutes at 37°C and was neutralised by adding 0.1 volumes of 3M sodium acetate, pH 5. The DNA was precipitated with 4 volumes of ethanol for 15 minutes at -70°C and then pelleted by centrifugation at 14 000 x g for

10 minutes. The pelleted DNA was washed with 70% ethanol and after a second centrifugation, the pellet was dried under vacuum. The DNA was then redissolved in water to be used for sequencing analysis (Section 3.2.11).

3.2.11 Sequencing

The nucleotide sequence of the subcloned insert was determined according to the dideoxynucleotide chain termination method of Sanger *et al* (1977) using a commercially available sequencing kit (Sequenase; United States Biochemical Corporation, Cambridge, UK) and ^{35}S-dATP (Amersham). The annealing reaction comprised 7µl of denatured plasmid DNA, 2µl of Sequenase reaction buffer and 1µl primer (approximately 300ng). The tubes were warmed to 65°C for 2 minutes then allowed to cool slowly to <30°C. Dimethyl sulphoxide (DMSO) was added to the termination mixes at a ratio of 9:1 (mix:DMSO) and also to the dilute labelling mix in the same ratio (Winship, 1989). The labelling reaction was carried out by adding 1µl dithiothreitol (DTT, 0.1M), 2µl diluted labelling mix (1:4), 0.5µl ^{35}S-ATP and 2 µl diluted Sequenase enzyme (1:8) to the annealed template-primer mix and this was incubated at room temperature for 5 minutes. The reactions were terminated by adding 3.5µl of the sequencing reaction mixture to each of the prewarmed (37°C) termination mixes containing a dideoxynucleotide (ddA, ddC, ddG and ddT; 2.5µl) and incubated for 5 minutes at 37°C. The reactions were finally stopped by adding 4µl of formamide stop dye to each termination mix and the vials placed on ice until the gel was prepared. The sequencing reaction products were run on a Hydrolink Long Ranger sequencing gel (AT Biochem, Newcastle, Staffs, UK) prepared according to the manufacturer's instructions. Gels were run using an LKB power supply at 35 W constant power for 3 hours. The gel was transferred to Whatman 3MM chromatography filter paper and carefully covered with cling film. The gel was dried

under vacuum at 80°C for 30 minutes (slab Gel Dryer, Hoefer Scientific Instruments, Newcastle, Staffs, UK) after which the cling film was removed and the gel exposed to X-ray film (Kodak X-OMAT AR; Kodak, Liverpool, UK) overnight. The X-ray film was developed after 24-48 hours using X-ray film developer and fixer (Kodak liverpool, UK). Analysis of human and marmoset polypeptide sequence was done by GeneJockey software (Medical Research Council, UK) while the hydropathicity profile of marmoset ZP3 polypeptide chain was created by Kyte-Doolittle computer analysis (Kyte and Doolittle, 1982).

3.2.12 Restriction Map Analysis

The sequenced marmoset ZP3 gene was restriction map analysed using Genejockey software (Medical Research Council, UK).

3.3 Results

The marmoset poly(A)+ RNA isolated from marmoset ovarian tissue was used to create marmoset ovarian cDNA which, in turn, served as a substrate for the amplification of ZP3 cDNA. Due to the difficulty in obtaining larger PCR fragments, we chose to amplify marmoset ZP3 exon 1-6 and exon 4-8 separately using primers based on the human ZP3 sequence (Chamberlin & Dean, 1990). The PCR products were electrophoresed on an 0.8 % agarose gel (Figure 3. 2) which indicated that the PCR product containing marmoset ZP3 exon 1-6 was approximately 0.8 kb in size. Marmoset ZP3 exon 4-8 PCR products were electrophoresed on an 0.8 % agarose gel (Figure 3. 3) which indicated that the PCR product was approximately 0.6 kb in size. Both cDNA fragments, encoding exon 1-6 and 4-8 respectively were cloned into the PCR 1000 vector. Positive white colonies containing marmoset ZP3 exon 1-6 in PCR

1000 vector were identified by restriction enzyme digestion with Nco1 restriction enzyme. The positive plasmid contained approximately a 750nt band of marmoset exon 1-6 and a PCR 1000 vector band of 600nt (Figure 3. 4). Positive white colonies containing marmoset exon 4-8 in PCR 1000 vector were identified by restriction enzyme digestion with Nco1 and Sac1. The positive plasmid released 550 nt bands are shown in Figure 3.5. The plasmids containing marmoset ZP3 exons 1-6 and 4-8 were sequenced in both directions to exclude the possibility of PCR-induced mutations. Moreover, the validity of the sequence data was confirmed on 4 independent analyses.

The entire cloned marmoset ZP3 cDNA sequence is presented in Figure 3.7. MarZP3 possesses an open reading frame of 1272 nucleotides, which is identical in length to the published human ZP3 sequence (Chamberlin & Dean, 1990). Similarities to the human ZP3 sequence also exist in that the marmoset ZP3 mRNA does not have a 3'untranslated region, since the translational stop codon TAA coincides with the RNA polyadenylation signal (Chamberlin & Dean, 1990). A partial restriction map of mar ZP3 cDNA is presented in Figure 3.6.

The predicted polypeptide consists of 424 amino acids, with a calculated molecular mass of 46815. An alignment with the human, mouse and hamster sequences is presented in Figure 3.8. The primary amino acid structure revealed a significant similarity between the marZP3 and huZP3 (Chamberlin & Dean, 1990), giving an overall homology of about 91% (Figure 3.8). The disparity between these proteins is confined to widely dispersed changes in one or two amino acids with the exception of the polypeptide chain spanning residues 322-352 in which the homology was reduced to 45%. Comparison of marZP3 with the murine and hamster ZP3 sequences indicated an overall homology of 68% and 61% respectively.

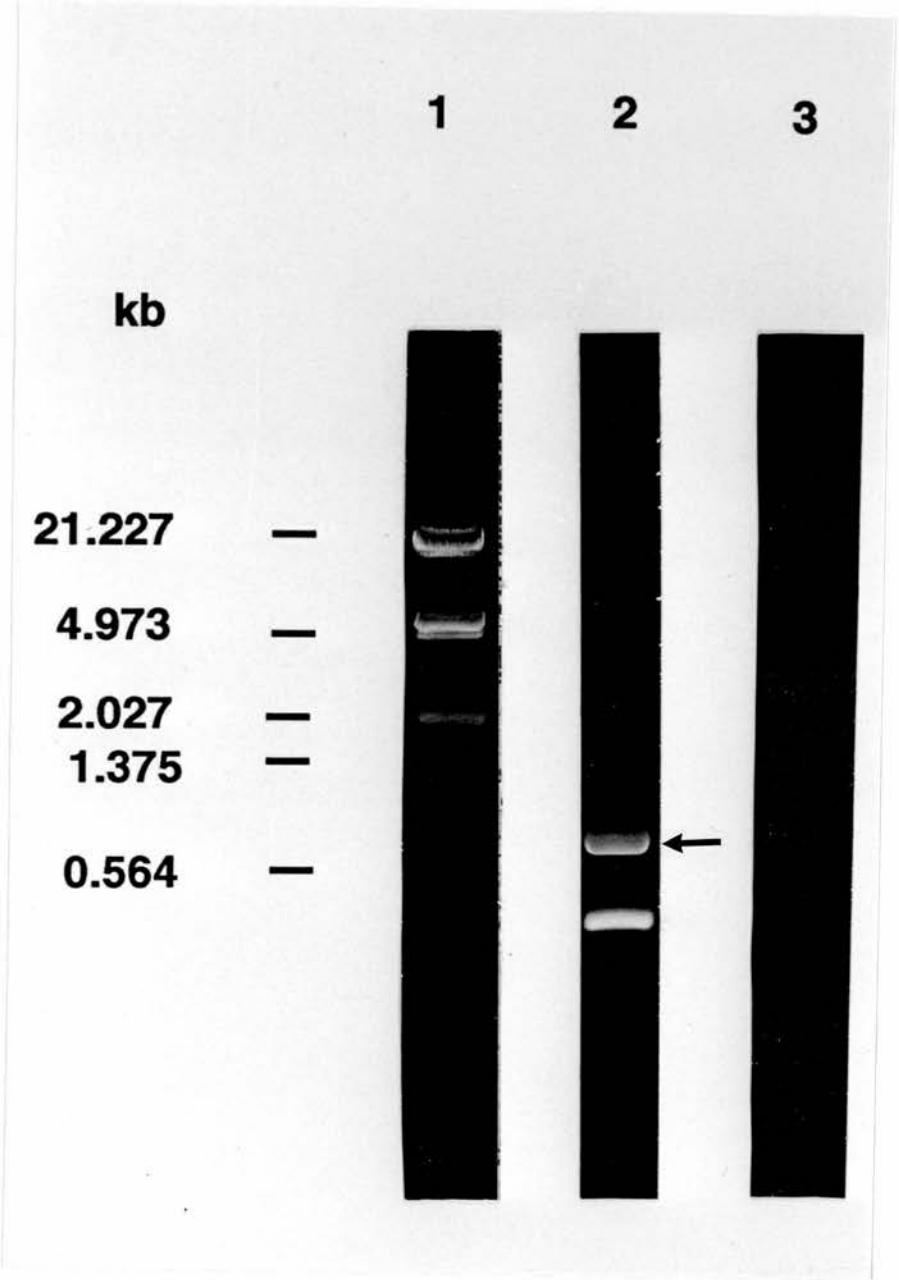


Figure 3.2 Electrophoresis in 0.8% agarose gel showing PCR products. Lane 1, DNA size markers, an *EcoRI* and *Hind III* digest of lamda DNA producing bands of sizes 21 227bp, 5148bp, 4973bp, 4268bp, 3530bp, 2027bp, 1904bp, 1584bp, 1375bp, 975bp, 831bp, 564bp and 125bp; Lane 2, PCR fragment of mar ZP3 exon 1-6, which is indicated by an arrow; Lane 3, same as lane 2 but no substrate was added. The gel was run at 80V for 1-2 hour in 0.5 x TBE.

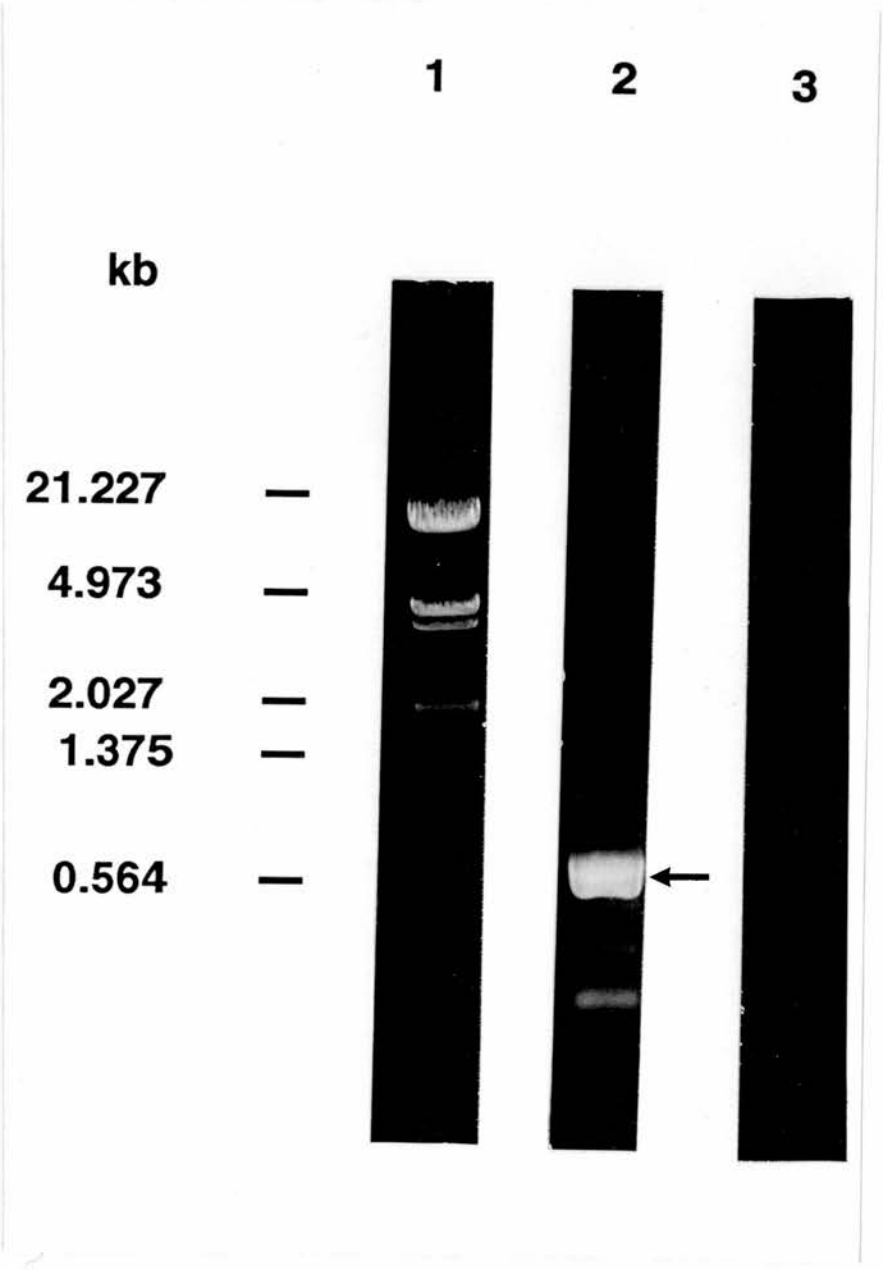


Figure 3.3 Electrophoresis in 0.8% agarose gel showing PCR products. Lane 1, DNA size markers, an *EcoRI* and *Hind III* digest of lamda DNA; Lane 2, PCR fragment of mar ZP3 exon 4-8, which is indicated by an arrow; Lane 3, same as lane 2 but no substrate was added. The gel was run at 80V for 1-2 hour in 0.5 x TBE.

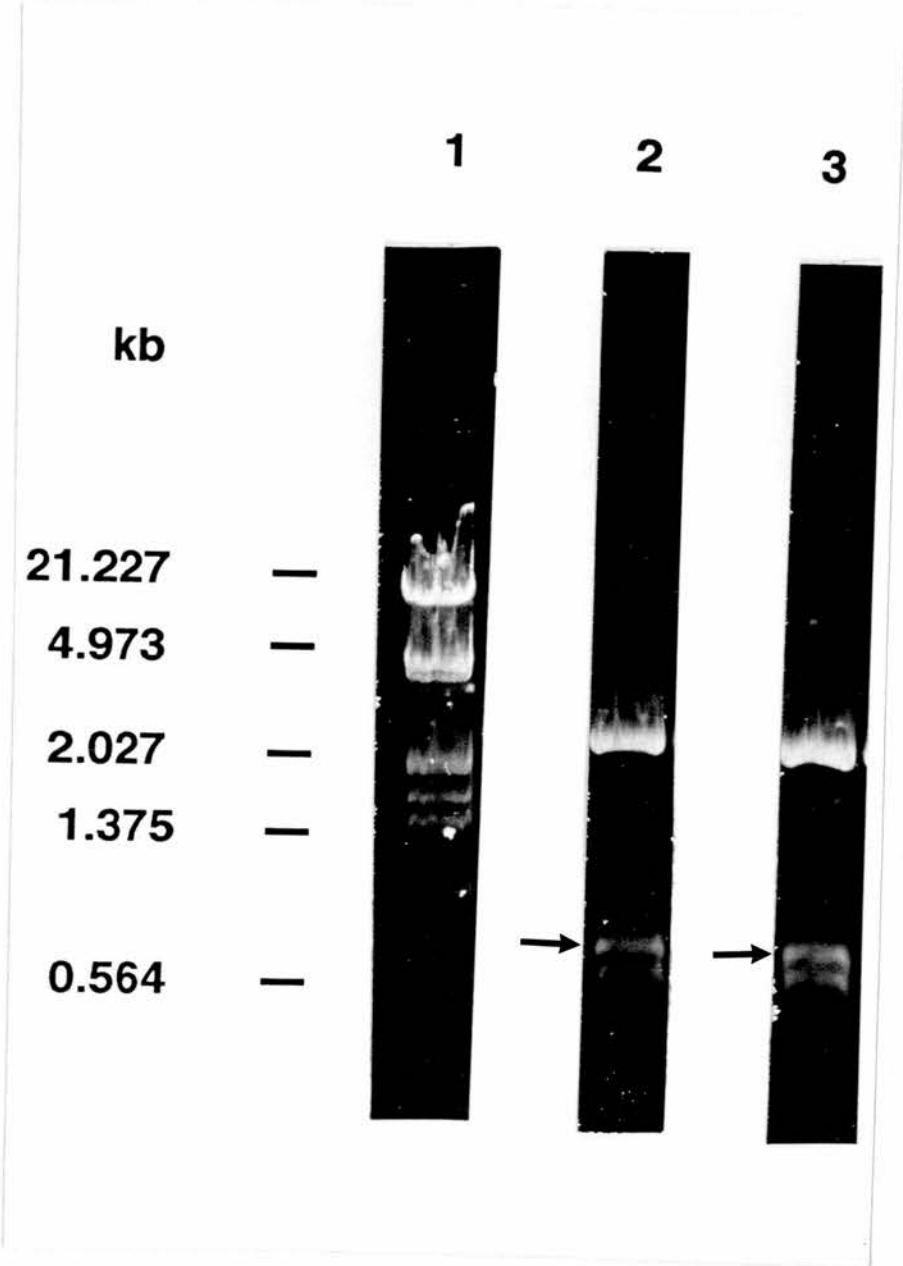


Figure 3.4 Electrophoresis in 0.8% agarose gel. Positive white colonies containing mar ZP3 exon 1-6 in PCR 1000 vector digested with Nco1 restriction enzyme. Lane 1, DNA size markers, an *EcoRI* and *Hind III* digest of lamda DNA; Lane 2, positive clone digested with Nco1 showing release of exon 1-6 insert of approximately 750 bp indicated by an arrow and PCR 1000 vector band of size 600 bp. Lane 3, same as lane 2. The gel was run at 80V for 1-2 hour in 0.5 x TBE.

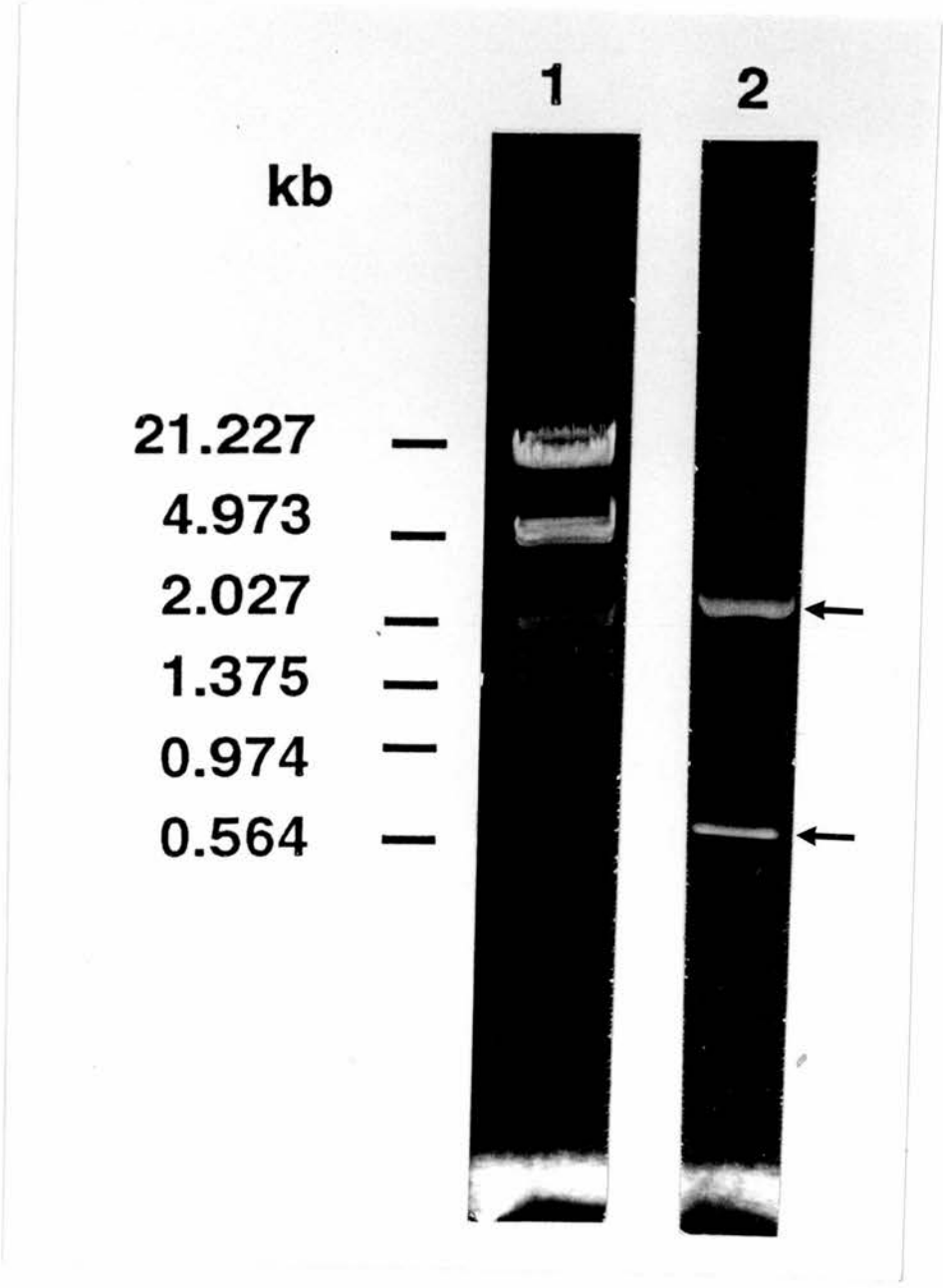


Figure 3.5 Electrophoresis in 0.8% agarose gel. Positive white colonies containing mar ZP3 exon 4-8 in PCR 1000 vector digested with Sac1 and Nco1 restriction enzymes. Lane 1, DNA size markers, an *EcoRI* and *Hind III* digest of lamda DNA; Lane 2, positive PCR 1000 vector digested with Sac1 and Nco1 showing release of exon 4-8 insert of approximately 550bp indicated by an arrow and the PCR1000 vector band of 3000bp, which is also indicated by an arrow. The gel was run at 80V for 1-2 hour in 0.5 x TBE.

Human, marmoset, hamster and mouse ZP3 contain 4, 5, 4, and 6 potential sites for N-linked glycosylation respectively (Struck & Lennarz, 1980; Kornfeld & Kornfeld, 1985) of which two positions are conserved between all species (Figure 3.8) while the marmoset and human ZP3 share 4 sites for the addition of N-linked carbohydrate side chains.

As in the other species for which the amino acid sequence is known, marZP3 is characterized by an abundance of serine and threonine residues, 90% of which are conserved in the human ZP3 protein. All 15 cysteine residues (Chamberlin & Dean, 1990) present in both human and marmoset ZP3 molecules are located in the same positions, where they may participate in the formation of disulphide bonds to maintain the secondary structure of the ZP3 proteins. Proline residues (29) were also unusually common in both marZP3 and huZP3 and were conserved with the exception of residue 305 in the human and residue 347 in the marmoset sequence. A hydrophobicity plot (Figure 3.9) of marZP3 showed the presence of one hydrophobic sequence at the amino terminus and two hydrophobic segments at the carboxyl terminal of the protein sequence. Comparison of huZP3 and marZP3 hydropathicity plots generated similar profiles (Figure 3 9). On the basis of protein sequence homology and hydrophobicity profile, a 22 amino acid signal peptide was predicted at the amino terminus of marZP3 (Von Heijne, 1986) giving a secreted peptide of 402 amino acids with a molecular mass of 44182 D and an N-terminal glutamine.

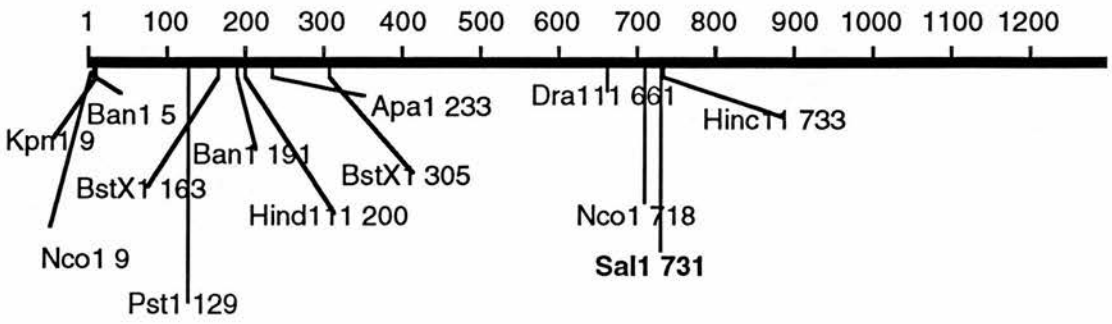


Figure 3.6 Partial restriction map of marmoset ZP3 cDNA analysed using Genejockey software (Medical Research Council, UK). Sal 1 restriction enzyme site is indicated in bold letters, which was employed in subcloning and creating a full length mar ZP3 cDNA.

```
   1                                                          5'-TGCAGGTACC
      Met  Glu  Leu  Ser  Tyr  Arg  Leu  Phe  Ile  Cys  Leu  Leu  Leu  Trp  Gly  Ser  16
  11 ATG  GAG  CTG  AGC  TAT  AGG  CTA  TTC  ATC  TGC  CTC  CTG  CTC  TGG  GGT  AGT
      Thr  Glu  Leu  Cys  Tyr  Pro  Gln  Pro  Leu  Arg  Leu  Leu  Gln  Gly  Gly  Thr  32
  60 ACT  GAG  CTG  TGC  TAC  CCC  CAA  CCC  CTC  AGG  CTC  TTA  CAG  GGT  GGA  ACC
      Ser  His  Pro  Glu  Thr  Ala  Leu  Gln  Pro  Val  Val  Val  Glu  Cys  Gln  Glu  48
 108 AGC  CAC  CCT  GAG  ACC  GCT  CTG  CAG  CCC  GTA  GTG  GTG  GAG  TGT  CAG  GAA
      Ala  Thr  Leu  Val  Val  Thr  Val  Ser  Lys  Asp  Leu  Phe  Gly  Thr  Arg  Lys  64
 156 GCC  ACC  CTA  GTG  GTC  ACA  GTC  AGT  AAA  GAC  CTT  TTT  GGC  ACC  AGG  AAG
      Leu  Ile  Arg  Ala  Val  Asp  Leu  Thr  Leu  Gly  Pro  Glu  Gly  Cys  Glu  Pro  80
 204 CTT  ATC  AGG  GCT  GTT  GAT  CTC  ACC  CTG  GGC  CCA  GAG  GGC  TGT  GAG  CCC
      Leu  Val  Ser  Thr  Asp  Thr  Glu  Asp  Val  Val  Arg  Phe  Glu  Val  Gly  Leu  96
 252 CTG  GTC  TCC  ACG  GAC  ACA  GAG  GAT  GTG  GTC  AGG  TTT  GAG  GTT  GGA  CTC
      His  Glu  Cys  Gly  Asn  Ser  Met  Gln  Val  Thr  Asp  Asp  Ala  Leu  Val  Tyr  112
 300 CAT  GAG  TGT  GGT  AAC  AGC  ATG  CAG  GTG  ACC  GAC  GAT  GCC  CTG  GTG  TAC
      Ser  Thr  Phe  Leu  Leu  His  Asp  Pro  Arg  Pro  Val  Gly  Asn  Leu  Ser  Ile  128
 348 AGC  ACC  TTC  CTG  CTT  CAC  GAC  CCC  CGC  CCT  GTG  GGA  AAC  CTG  TCC  ATC
      Val  Arg  Thr  Asn  Arg  Ala  Glu  Ile  Pro  Ile  Glu  Cys  Arg  Tyr  Pro  Arg  144
 396 GTG  AGG  ACT  AAC  CGC  GCA  GAG  ATT  CCC  ATC  GAG  TGC  CGC  TAC  CCC  AGG
      Arg  Gly  Asn  Val  Ser  Ser  Gln  Ala  Ile  Leu  Pro  Thr  Trp  Leu  Pro  Phe  160
 444 CGG  GGC  AAT  GTG  AGC  AGC  CAG  GCC  ATC  CTT  CCC  ACC  TGG  CTG  CCC  TTC
      Arg  Thr  Thr  Val  Phe  Ser  Glu  Glu  Lys  Leu  Thr  Phe  Ser  Leu  Arg  Leu  176
 492 AGG  ACC  ACG  GTG  TTC  TCA  GAG  GAG  AAG  CTG  ACT  TTC  TCT  CTG  CGC  CTG
      Met  Glu  Glu  Asn  Trp  Ser  Thr  Glu  Lys  Arg  Thr  Pro  Thr  Phe  His  Leu  192
 540 ATG  GAG  GAG  AAC  TGG  AGC  ACT  GAG  AAG  AGG  ACC  CCT  ACC  TTC  CAC  CTG
      Gly  Asp  Val  Ala  His  Leu  Gln  Ala  Glu  Ile  His  Thr  Gly  Ser  His  Val  208
 588 GGA  GAT  GTG  GCC  CAC  CTC  CAG  GCA  GAA  ATC  CAC  ACT  GGC  AGC  CAC  GTG
      Pro  Leu  Arg  Leu  Phe  Val  Asp  His  Cys  Val  Ala  Thr  Pro  Thr  Pro  Asp  224
 636 CCA  CTG  CGG  CTA  TTT  GTG  GAC  CAC  TGT  GTG  GCC  ACG  CCA  ACA  CCA  GAC
      Gln  Asn  Ala  Ser  Pro  Tyr  His  Thr  Ile  Val  Asp  Phe  His  Gly  Cys  Leu  240
 684 CAG  AAT  GCC  TCC  CCT  TAT  CAC  ACC  ATC  GTG  GAC  TTC  CAT  GGC  TGT  CTT
      Val  Asp  Gly  Leu  Thr  Asp  Ala  Ser  Ser  Ala  Phe  Gln  Ala  Pro  Arg  Pro  256
 732 GTC  GAC  GGT  CTC  ACT  GAT  GCC  TCT  TCT  GCA  TTC  CAA  GCT  CCC  AGA  CCC
      Arg  Pro  Asp  Thr  Leu  Gln  Phe  Thr  Val  Asp  Val  Phe  His  Phe  Ala  Asn  272
 780 AGG  CCA  GAT  ACA  CTC  CAG  TTC  ACG  GTG  GAT  GTG  TTT  CAT  TTT  GCT  AAT
      Asp  Ser  Arg  Asn  Met  Ile  Tyr  Ile  Thr  Cys  His  Leu  Lys  Val  Thr  Leu  288
 828 GAC  TCC  AGA  AAT  ATG  ATA  TAC  ATC  ACC  TGC  CAC  CTG  AAG  GTC  ACC  CTA
      Ala  Glu  Gln  Asp  Pro  Asp  Glu  Leu  Asn  Lys  Ala  Cys  Ser  Phe  Ser  Lys  304
 876 GCT  GAG  CAG  GAC  CCA  GAT  GAA  CTG  AAC  AAA  GCC  TGT  TCC  TTC  AGC  AAG
      Ala  Ser  Asn  Ser  Trp  Phe  Pro  Val  Glu  Gly  Pro  Ala  Asp  Ile  Cys  Gln  320
 924 GCT  TCC  AAC  AGC  TGG  TTC  CCG  GTG  GAA  GGC  CCG  GCT  GAC  ATC  TGC  CAG
      Cys  Cys  Ser  Lys  Gly  Asp  Cys  Gly  Thr  Pro  Ser  His  Ala  Arg  Arg  Gln  336
 972 TGC  TGT  AGC  AAG  GGT  GAC  TGT  GGC  ACT  CCA  AGC  CAT  GCC  AGG  AGG  CAG
      Pro  His  Val  Val  Ser  Leu  Gly  Ser  Gly  Ser  Pro  Ala  Arg  Asp  Arg  Arg  352
1020 CCC  CAT  GTC  GTG  AGC  CTG  GGG  TCG  GGT  TCT  CCT  GCC  CGT  GAC  CGC  AGG
      His  Val  Thr  Glu  Glu  Ala  Asp  Val  Thr  Val  Gly  Pro  Leu  Ile  Phe  Leu  368
1068 CAT  GTG  ACA  GAA  GAA  GCA  GAC  GTC  ACC  GTG  GGA  CCG  CTG  ATC  TTC  CTG
      Asp  Arg  Thr  Gly  Asp  His  Glu  Met  Glu  Gln  Trp  Ala  Leu  Pro  Ala  Asp  384
1116 GAC  AGG  ACT  GGT  GAC  CAC  GAA  ATG  GAG  CAG  TGG  GCC  TTG  CCG  GCT  GAC
      Thr  Ser  Leu  Leu  Leu  Leu  Gly  Thr  Gly  Leu  Ala  Val  Val  Ala  Leu  Leu  400
1164 ACC  TCC  TTG  CTG  CTG  CTG  GGC  ACA  GGC  CTG  GCT  GTT  GTG  GCG  CTC  CTG
      Thr  Leu  Thr  Ala  Val  Ile  Leu  Val  Leu  Thr  Arg  Arg  Cys  Arg  Thr  Ala  416
1212 ACT  CTG  ACC  GCT  GTT  ATC  CTG  GTT  CTC  ACC  AGG  AGG  TGT  CGC  ACT  GCC
      Ser  Leu  Pro  Val  Ser  Ala  Ser  Glu  Stop                                  424
1260 TCC  CTC  CCT  GTG  TCT  GCT  TCC  GAA  TAA  AAGAAGAAAGCAAT-3'
```

Figure 3.7 The top line is the marmoset mRNA from marmoset cDNA sequences and the bottom line is the aminoacid sequences translated from the single open reading frame. The signal peptide sequence (1-22 amino acids) is italicized and the potential N-linked glycosylation sites are underlined. The primers used for the PCR are indicated in bold letters.

```
Human       MELSYRLFICLLLWGSTELCYPQPLWLLQGGASHPETSVQPVLVECQEAT   50
Marmoset    MELSYRLFICLLLWGSTELCYPQPLRLLQGGTSHPETALQPVVVECQEAT   50
Mouse       MASSYFLFLCLLLCGGPELCNSQTLWLLPGGTPTPVGSSSPVKVECLEAE   50
Hamster     MGLSYQLLLCLLLCGGAKQCCSQPLWLLPGGTPTPGKLTSSVEVECLEAE   50
            * ·** *··**** *··· * ·*·*·**·**·· *     ·* *** **·

Human       LMVMVSKDLFGTGKLIRAADLTLGPEACEPLVSMDTEDVVRFEVGLHECG   100
Marmoset    LVVTVSKDLFGTRKLIRAVDLTLGPEGCEPLVSTDTEDVVRFEVGLHECG   100
Mouse       LVVTVSRDLFGTGKLVQPGDLTLGSEGCQPRVSVDT-DVVRFNAQLHECS   99
Hamster     LVVIVSRDLFGTGKLIQPEDLTLGSENCRPLVSVAT-DVVRFKAQLHECS   99
            *·* **·**** **··· *******·*· * ** ·* ******·· ****·

Human       NSMQVTDDALVYSTFLLHDPRPVGNLSIVRTNRAEIPIECRYPRQGNVSS   150
Marmoset    NSMQVTDDALVYSTFLLHDPRPVGNLSIVRTNRAEIPIECRYPRRGNVSS   150
Mouse       SRVQMTKDALVYSTFLLHDPRPVSGLSILRTNRVEVPIECRYPRQGNVSS   149
Hamster     NRVQVTEDALVYSTVLLHQPRPVPGLSILRTNRADVPIQCRYPRQGNVSS   149
            ···*·*·******* ***·**** ·***·*****···**·*****·*****

Human       QAILPTWLPFRTTVFSEEKLTFSLRLMEENWNAEKRSPTFHLGDAAHLQA   200
Marmoset    QAILPTWLPFRTTVFSEEKLTFSLRLMEENWSTEKRTPTFHLGDVAHLQA   200
Mouse       HPIQPTWVPFRATVSSEEKLAFSLRLMEENWNTEKSAPTFHLGEVAHLQA   199
Hamster     HAIRPTWVPSRTTVSSEEKLVFSLRLMEENWNTEKLSPTSHLGEVAYLQA   199
            ··* ***·* *·** *****·**********·** ·** ***··*·***

Human       EIHTGSHVPLRLFVDHCVATPTP--DQNASPYHTIVDFHGCLVDGLTDAS   248
Marmoset    EIHTGSHVPLRLFVDHCVATPTP--DQNASPYHTIVDFHGCLVDGLTDAS   248
Mouse       EVQTGSHLPLQLFVDHCVATPSPLPDPNSSPYHFIVDFHGCLVDGLSESF   249
Hamster     EVQTGSHLPLLLFVERCVATPS--PDQTASPYHVIVDFHGCLVDGLSESF   247
            *··*****·** ***··*****·   *···**** ************···

Human       SAFKVPRPGPDTLQFTVDVFHFANDSRNMIYITCHLKVTLAEQDPDELNK   298
Marmoset    SAFQAPRPRPDTLQFTVDVFHFANDSRNMIYITCHLKVTLAEQDPDELNK   298
Mouse       SAFQVPRPRPETLQFTVDVFHFANSSRNTLYITCHLKVAPANQIPDKLNK   299
Hamster     SAFQVPRPRPETLQFTVDVFHFANSSRNTIYITCHLKVTPANQTPDELNK   297
            ***··*** *·**************·*** ·********· *·* **·***

Human       ACSFSKPSNSWFPVEGPADlCQCCNKGDCGTPSHSRRQPHVMSQWSRSAS   348
Marmoset    ACSFSKASNSWFPVEGPADICQCCSKGDCGTPSHARRQPHVVSLGSGSPA   348
Mouse       ACSFNKTSQSWLPVEGDADICDCCSHGNCSNSSSSQFQIHGPRQWSKLVS   349
Hamster     ACSFNRSSKSWSPVEGDAEVCGCCSSGDCGSSSRSRYQAHGVSQWPKSAS   347
            **********·** **** *·*· **· *·*···· .. * *  ·  ·   ·

Human       RNRRHVTEEADVTVGPLIFLDRRGDHEVEQWALPSDTSVVLLGVGLAVVV   398
Marmoset    RDRRHVTEEADVTVGPLIFLDRTGDHEMEQWALPADTSLLLLGTGLAVVA   398
Mouse       RNRRHVTDEADVTVGPLIFLGKANDQTVEGWTASAQTSVAL-GLGLATVA   398
Hamster     RRRRHVRDEADVTVGPLIFLGKASDQAVEGWASSAQTSLAL-GLGLAAVA   396
            * **** ·************·· ·*···* *· ····*· * * ***·*·

Human       SLTLTAVILVLTRRCRTASHPVSASE 424
Marmoset    LLTLTAVILVLTRRCRTASLPVSASE 424
Mouse       FLTLAAIVLAVTRKCHSSSYLVSLPQ 424
Hamster     FLTLAAIVLGVTRSCHTPSHVVSLSQ 422
             *******·* ·*******·*  **·
```

Figure 3.8 Comparison of the primary amino acid sequence of ZP3 for the four species. Asterisks indicate conserved amino acids and dots indicate highly conserved amino acids. Potential N-linked glycosylation sites (NXT or NXS) are underlined.

3.4 Discussion

In this study we have succeeded in elucidating the primary amino acid sequence of marmoset ZP3 employing a PCR strategy to amplify marZP3 cDNA, which has then been cloned and sequenced. Mar ZP3 has a single open reading frame 1272 nucleotides in length, which gives rise to a polypeptide core of 424 amino acids. This is exactly the same size as human and mouse ZP3 (Chamberlin & Dean, 1990) and 2 amino acids longer than the hamster homologue (Kinloch *et al.*1990). On the basis of the hydrophobicity plot (Kyte & Doolittle, 1982) and amino acid homology, a signal peptide of 22 amino acids could be identified, the four amino acids near the cleavage site sharing the "-3, -1 rule" for predicting signal sequence cleavage sites (Von Heijne, 1986). In this respect marmoset ZP3 is identical to the other species in which the primary amino acid structure is known including the mouse (Kinloch *et al.* 1988; Ringuette *et al.* 1988; Chamberlin and Dean, 1989) hamster (Kinloch *et al.* 1990; Moller *et al.* 1990) and human (Chamberlin and Dean, 1990; Van Duin et al., 1992). The marZP3 also shared with the other species in which this molecule has been sequenced, a second broad hydrophobic domain near the carboxyl terminus, the function of which is currently unknown.

The primary amino acid structure of marZP3 was found to exhibit a high degree of homology (91%) with the huZP3 sequence and more than 60% homology with the mouse and hamster sequences. Intriguingly, the most significant differences between the human and marmoset sequences were confined to a short stretch of amino acids spanning residues 322-352, over which the level of homology was reduced to 45%. A region of the ZP3 sequence covering residues 318 to 352, was also found to house the greatest sequence disparity when the mouse and hamster ZP3 sequences were compared (Kinloch *et al.* 1990). The existence of such a highly variable region

within an otherwise conserved ZP3 primary structure, might suggest a role for this domain in determining the species specificity of sperm-zona interaction.

Other characteristic features of the ZP3 molecule that were retained by the marZP3 molecule included the unusually high frequency of serine/threonine and proline residues, which have been found in all ZP3s sequenced to date. The latter comprised: (i) a region spanning amino acid 119 to 158 that contained 7 proline residues in the mouse and hamster and 6 in human and marmoset ZP3 and (ii) a second proline rich domain spanning residues 208-257 and containing 7 proline residues in the mouse and hamster and 5 in human and marmoset ZP3. An additional conserved feature of the ZP3 molecule is the number and location of the cysteine residues; the human, marmoset, mouse and hamster ZP3 sequences all possessing 15 cysteine residues at identical sites. Such a high level of conservation suggests a key role for intramolecular disulphide bonds in preserving the structural features of the polypeptide chain that are involved in the expression of biological activity.

Elucidation of the primary structure of marZP3 has not only been of value in emphasizing the way in which the structural features of this molecule have evolved in different species but will also be of considerable importance in the development of contraceptive vaccines. By demonstrating the considerable sequence homology that exists between the human and marmoset ZP3, the way is now open to develop a clinically-relevant active immunization model for evaluating the influence of anti-zona antibodies on reproductive function, employing antigens based on homologous regions of the ZP3 polypeptide chain.

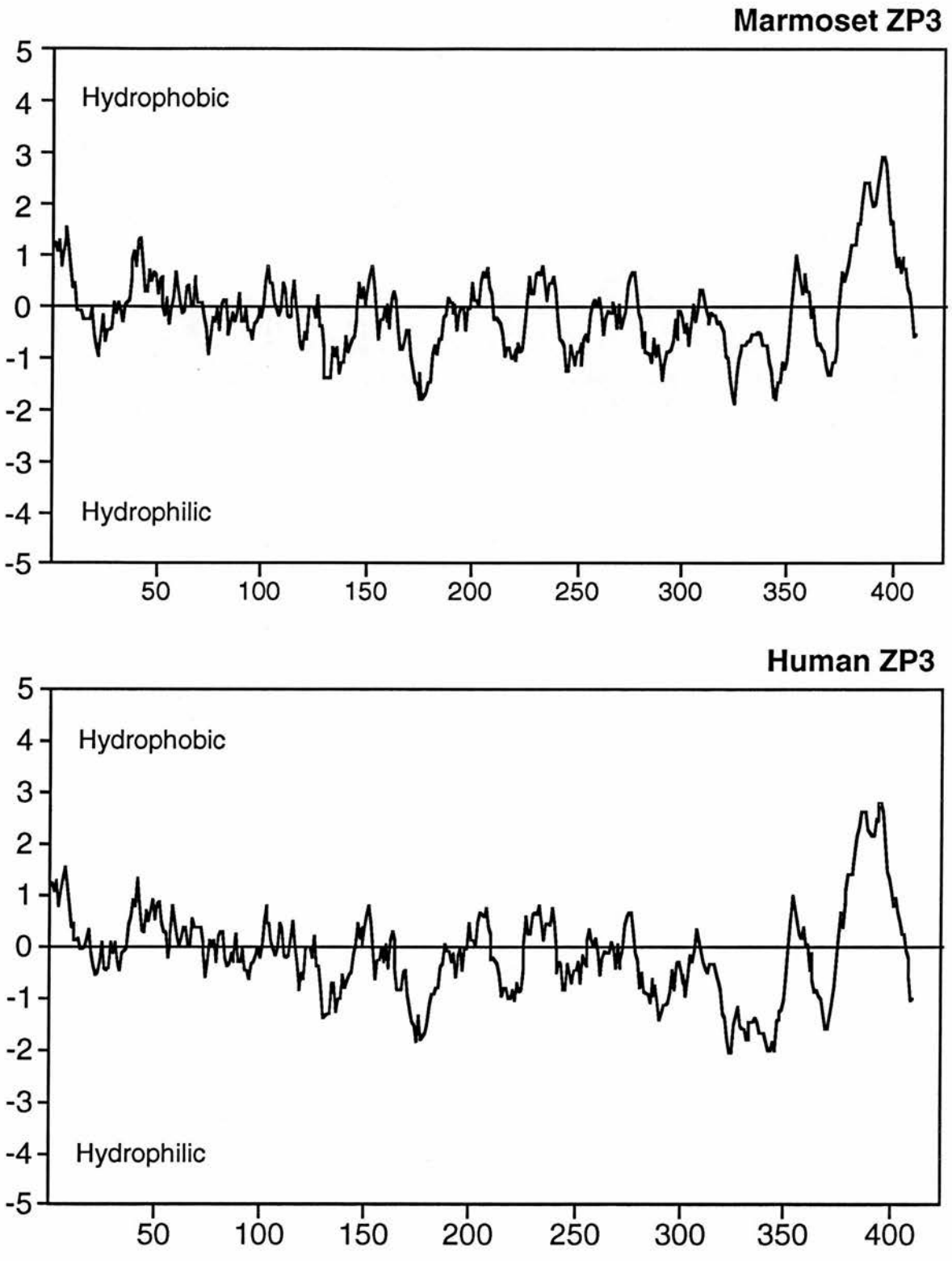


Figure 3.9 Hydropathicity profiles of marmoset and human ZP3 determined by the Kyte and Doolittle (1982) algorithm, showing the conserved nature of the two ZP3 proteins.

3.5 Summary

A PCR strategy was used to amplify, clone and sequence marmoset ZP3 cDNA. The primary amino acid structure of marZP3 was found to exhibit a high degree of homology (91%) with the huZP3 sequence and more than 60% homology with the mouse and hamster sequences. An additional conserved feature of the ZP3 molecule is the number and location of the cysteine residues; the human, marmoset, mouse and hamster ZP3 sequences all possessing 15 cysteine residues at identical sites. Elucidation of mar ZP3 primary amino acid sequence has been of value in emphasizing the evolution and conservation of sperm receptor structure in different species.

4 Oocyte-Specific Expression of Marmoset ZP3

4.1 Introduction

At birth the mouse ovary contains 10,000 to 15,000 primordial oocytes (Jones & Krohn, 1961), many of which are in the prophase of the first meiotic division, the resting condition known as dictyate. At recruitment, resting oocytes of 10 to 15 μm in diameter enter into a 2-week growth phase which culminates in meiotic maturation and subsequent ovulation (Baker, 1972; Brambell, 1928). The completion of meiosis as evidenced by the exclusion of the second polar body does not occur until the oocyte has been fertilized in the oviduct (Eppig, 1985; Bachvarova, 1985). Nothing is known about the regulatory elements, and their signals, that might induce the growth and differentiation of oocytes, although secretion of the ZP is thought to be an important sign that activation of the oocyte has occurred on recruitment to the primary follicle pool (Philpott *et al.*1987; Liang *et al.*1990; Roller *et al.*1989; Lira *et al.*1990).

Although the biochemical constituents of the ZP have been well characterized (Wassarman, 1987b; Wassarman, 1988a; Wassarman, 1988b; Shimizu *et al.*1983), controversy has arisen as to whether the ZP proteins are synthesised by the oocytes and/or by the granulosa cells. Some of the early studies suggested that ZP proteins were synthesised by growing oocytes as well as the granulosa cells of the ovary (Wolgemuth *et al.*1984). Antibodies developed against total ZP proteins were employed to localise the formation of ZP proteins in the ovary. The ZP proteins were first observed in the cytoplasm and at the periphery of the oocytes, surrounded by a thin squamous follicular cell layer. As the ZP matrix was assembled extracellularly,

the intensity of staining of the outer and inner regions could be distinguished. (Wolgemuth *et al.*1984). Radiolabelled antibodies or immunofluorescence techniques have been employed to monitor the distribution of anti-zona monoclonal antibodies in ovarian tissue sections and in all cases the immunoreactivity has been found to target the ZP exclusively (Dean & East, 1986; East *et al.*1984a; East & Dean, 1984). Furthermore, biosynthetic studies of the mouse ZP using an *in vitro* culture system clearly revealed that the ZP proteins were synthesised in growing oocytes (Shimizu *et al.*1983; Bleil & Wassarman, 1980c). These findings have been reinforced by the use of powerful tools like *in situ* hybridisation (Philpott *et al* 1987) Thus a DNA construct that placed the hZP3 gene under the control of mZP3 gene 5'-flanking sequence was used to produce two lines of transgenic mice that harboured the foreign sperm receptor transgene. The transgene was expressed only by growing oocytes, at a level comparable to that of the endogenous mZP3 gene, and the developmental pattern of transgene expression resembled that of the mZP3 gene (Lira *et al.*1990).

In situ hybridisation has become a powerful tool in biology. The basis of *in situ* hybridisation is that a piece of labelled ribonucleic acid (RNA) or complementary deoxyribonucleic acid (cDNA) from a gene of interest can be hybridised to a section of tissue, permitting identification of the cells transcribing that particular gene. *In situ* hybridisation studies were initially performed to detect DNA targets and amplified ribosomal RNA within cell nuclei (Gall & Pardue, 1969; John *et al.*1969; Buongiorno-Nardelli & Amaldi, 1970). However recent improvements in cloning techniques have made available probes for a large and rapidly increasing number of mRNA species.

Work by Angerer and Angerer (1981) dealing with sea urchin embryo development using RNA probes, has given us the methodology for conducting *in situ* hybridization experiments. One significant problem is that the greater the size of the probe the more difficulties are encountered in achieving penetration of the tissue to be

examined. This problem could be circumvented either using hydrolysed shorter probes or oligonucleotide probes. Coghlan *et al* (1985) solved this difficulty by using synthetic oligonucleotide probes. Introduction of biotinylated probes in *in situ* hybridisation has been an additional advance which has made the approach safe from the danger of handling radioactivity (Singer & Ward, 1982).

The purpose of this study was to use the information we had gained on the structure of the marmoset ZP3 gene to design probes that could be labelled and used to examine the cellular distribution of mar ZP3 transcripts in the marmoset ovary.

4.2 Materials and Methods

4.2.1 Marmoset Genomic DNA Isolation

Marmoset liver DNA was collected for genomic DNA isolation from the Medical Research Council Primate Colony, Edinburgh. The marmoset liver was collected in liquid nitrogen and transported to the laboratory and stored at -80°C. Approximately 10g marmoset liver was crushed with a glass homogenizer in 40ml of extraction buffer (TE Buffer; 10mM Tris-HCl, 0.1M EDTA, pH 8.0; 20µg/ml Pancreatic-RNAse; 0.5% SDS). After one hour proteinase K (100µg/ml) was added and then kept at 50°C in a water bath for 3 hours. An equal volume of phenol:chloroform pH 8.0 was then added and the solution mixed to form an emulsion, then centrifuged at 4000 rpm for 15 minutes. The aqueous phase was withdrawn and the phenol:chloroform extraction was repeated twice as before. 2ml, 10M ammonium acetate and 44ml cold ethanol was then added to the 20ml aqueous phase . The DNA separated as a cloudy precipitate and was removed with a glass rod, washed with 70% ethanol and then pelleted by centrifugation. The DNA was left at room temperature to dry and then dissolved with TE buffer, pH 8.0.

4.2.2 PCR Amplification of Marmoset ZP3 Exon 8

The marmoset genomic DNA was used as a template to amplify marmoset ZP3 exon 8 using the polymerase chain reaction (PCR; Saiki *et al*, 1985, 1988; Mullis & Faloona, 1987). PCR was performed using a GeneAMP PCR Reagent kit with AmpliTaq DNA Polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) using two primers synthesised as described in Section 3.2.4 and a programmable heating block (Hybaid, Teddington, Middlesex, UK). Marmoset ZP3 exon 8, corresponding to the equivalent exon in human ZP3 (Chamberlin & Dean, 1990), was amplified with oligonucleotide primer 5, 5'-CACAGTGACAGAAGAAGCAG-3' and primer 6, 5'-AGATCTGAGCTCATTGCTTTCTTCTTTTATTCGGAAG-3' in 25 cycles of PCR. The final concentrations of the components in the 100µl reaction mixture were 2.5 Units of AmpliTaq DNA Polymerase, dNTPs, each 200µM; 1 x reaction buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin); 0.5 µM primer 1 and 0.5µM primer 2. The PCR reaction involved 25 cycles of incubation at 94°C for 2 minutes to achieve the initial melt, then 94°C for 1 minute, 60°C for 2 minutes, and 72°C for 3 minutes, followed by a final extension at 72°C for 15 minutes. The PCR reaction products were checked by electrophoresis in a submerged 1% agarose gel in 0.5 x TBE buffer run at a constant voltage of 80V for 1 hour.

4.2.3 Modification of Ragged Ends of PCR DNA

Based on the observation that Taq polymerase can add a single non template-directed deoxyadenosine residue to the 3'end of duplex PCR products, this non-template directed 3' end overhangs (ragged end) has to be blunt ended to clone the PCR DNA. The marmoset ZP3 exon 8 PCR product was sliced from the agarose gel and was purified from agarose gel electrophoresis using the Geneclean II kit (Geneclean II kit, Bio 101 Inc. La Jolla, USA). Approximately 500ng of a 230nt fragment of exon 8

marZP3, were dissolved in 10µl of 10 x kinase buffer 10µl (0.5 M Tris pH 7.5, 0.1 M $MgCl_2$, 10mM DTT, 0.5 mg/ml BSA, 200µM dNTPs), and to this mixture were added ATP 1 mM, and about 10 units each of T4 polynucleotide kinase and DNA polymerase (Clark, 1988; Aslanidis & de Jong, 1990). The final volume was adjusted to 100µl with water and incubated at 37°C for 1 hour, stopped with 1 µl of 0.5 M EDTA pH 8.0 and the blunt ended PCR amplified DNA purified using the Geneclean II kit.

4.2.4 Sub-cloning into Bluescript SK-

Bluescript SK- was digested with Sma1 restriction enzyme at 30°C for 3 hours. The reaction mixture consisted of 3µg of Bluescript SK- in 3µl, 5µl of BSA (1mg/ml), 5µl restriction buffer (10 x), restriction enzyme Sma1 (20 units) and the volume adjusted to 50µl with water. After 3 hours the Sma I cut plasmid was dephosphorylated to prevent self-ligation. The Sma1 cut plasmid DNA was mixed with 5µl, (10 x) calf intestine alkaline phosphate buffer, and calf intestine alkaline phosphatase (4 units) and the reaction was carried out at 37°C for 60 minutes. The reaction was stopped with 1µl of 0.5M EDTA and then the calf intestine alkaline phosphatase activity was heat-destroyed at 75°C for 10 minutes. The dephosphorylated plasmid DNA was phenol:chloroform extracted twice and then ethanol precipitated.

The ligation of Bluescript SK- with marmoset ZP3 exon 8 was carried out at 14°C overnight. The reaction mix contained 100ng Sma1 cut vector DNA, 100ng marmoset ZP3 exon 8 DNA, 4µl (5 x) ligase buffer, T4 DNA ligase (10 units), and water to make up the 20µl final volume. The following day, XL1-B competent cells (Section 4.2.5) were electrotransformed with this ligation mixture using a Gene Pulser (BioRad, Hemel Hempstead, Hertfordshire, UK) as follows. Aliquots of competent cells (40µl) were thawed at room temperature and then immediately placed on ice. 2µl

of the ligation mix was added to each cell suspension, mixed well and incubated on ice for 0.5-1 minute. The DNA-cell mixture was transferred to a cold 0.2 cm sterile electroporation cuvette and pulsed for 15 seconds. The Gene Pulser apparatus was set at 25μF and 2.5 kV and the Pulse Controller at 200 ohms. The cells were immediately resuspended in prewarmed (37°C) 1ml SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) and the cells incubated at 37°C for 1 hour with shaking in 17 x 100mm polypropylene tubes. The cells were then plated on LB plates containing 50μg/ml ampicillin, 40μg/ml X-gal and 0.5M IPTG (LAX plates) and grown overnight at 37°C. Cells transformed with recombinant plasmid result in white colonies. Several white (putative positive) colonies were grown up and plasmid DNA purified using Magic Mini-Prep columns (Promega, Southampton,UK). The purified DNA was checked by PCR (Section 4.2.2) and sequencing (3.2.11) to verify the presence of positive marmoset ZP3 exon 8.

4.2.5 Preparation of Competent Cells

E. coli XL1-Blue competent cells were prepared as follows. One litre of L broth was inoculated with 1/100 volume of a fresh overnight culture of XL1-B cells. The cells were grown at 37°C with vigorous shaking till the absorbency at 600nm was between 0.5 and 0.8. The cells were harvested by chilling the flask on ice for 15-30 minutes followed by centrifugation in a cold rotor at 4000 x g for 15 minutes. The supernatant was removed and the pellet resuspended in 1 litre of ice-cold water. The suspension was then centrifuged as before and the pellet resuspended in 0.5 litre of ice-cold water. After a third centrifugation, the pellet was resuspended in approximately 20ml of ice cold 10% glycerol. A final centrifugation step was performed and the cells resuspended in 2-3ml of ice-cold 10% glycerol so that the final concentration was

approximately 1-3 x 10^6 cells/ml. This suspension was frozen as 40µl aliquots on dry ice and stored at -70°C until required.

4.2.6 Riboprobe Preparation

DNA from Marmoset ZP3 exon 8 cloned in the Bluescript SK- was used for riboprobe preparation. DNA was digested with restriction enzyme BamHI to prepare the antisense probe, and restriction enzyme EcoRI for sense strand preparation. The digested DNA was phenol:chloroform extracted and then ethanol precipitated. BamHI digested DNA (1µg) was used for antisense riboprobe preparation, rATP, rGTP and rCTP each at 2.5mM, 2µl of 10 x transcription buffer, ribonuclease inhibitor (1unit), rUTP (å^{35}S 50µCi), T7 polymerase (20units) and water added to make the volume up to 20µl. The reaction was carried out at 37°C for 2 hours. EcoRI digested (1µg)DNA was used for sense strand DNA preparation and the reaction mix contained the same as the above antisense strand preparation, except that the transcription enzyme was T3 polymerase (20 units). After two hours of incubation, yeast tRNA, 2µl containing 10mg/ml, was added with RNAse-free DNAse (1unit) and incubated at 37°C for 10 minutes. The reaction was stopped with 1µl of 0.5M ETDA and the volume was increased to 100µl with the addition of 50mM DTT in TE buffer. The sense and antisense probe mix was phenol:chloroform extracted and then ethanol precipitated overnight.

4.2.7 Slide Preparation

Glass slides were washed and then baked in a hot air oven to destroy the RNAse enzyme. Washed slides were dipped in 2%, 3-aminopropyltriethoxysilane (Sigma, MO) in acetone and allowed to dry and the same procedure was repeated once again.

The slides were then rinsed in acetone and distilled water and the dried slides stored in boxes until use.

4.2.8 *In situ* Hybridization

Marmoset ovaries, stored at -80°C were used and 10µm sections were cut using a Cryostat. The sections were mounted on 3-aminopropyltriethoxysilane (Sigma, MO) coated slides (Van prooijen-Knegt *et al.*1982) for *in situ* hybridization as described above (Section 4.2.7). Ovarian sections were fixed in 4% paraformaldehyde, pH 7.4 for 5 minutes, washed in PBS (Flow Laboratories, Irvine, UK), pH 7.4 for 2 x 5 minutes, rinsed in water for 2 minutes, washed in 0.1 M triethanolamine pH 8.0, and incubated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 minutes (Angerer & Angerer, 1981). The RNA probes were added to a hybridization solution (50% deionised formamide, 10% dextran sulphate, 4x SSC (20x SSC = 3M sodium chloride/0.3M sodium citrate), 1x Denhardt's (1x Denhardt's solution = 0.02% Bovine serum albumin / 0.02% Ficoll / 0.02% Polyvinylpyrrolidone), 100 mM dithiothreitol, 125µg/ml yeast RNA, 125µg /ml salmon spermatozoa DNA) and 1 x 10^6 cpm/slide was applied to each tissue section before being overlaid with a coverslip. Slides were hybridized at 50°C for 16-18 hours and then the coverslips were removed and washed with 4 x SSC containing 4 mM dithiothreitol for 2 x 5 minutes. The slides were subsequently treated with RNAseA (30µg/ml) in 0.5M NaCl/10mM Tris-HCl/1mM EDTA, pH 8.0 at 37°C for 30 minutes. This wash was repeated without RNAse-A but containing 2mM dithiothreitol at 37°C for 30 min. The slides were then washed at 50°C in 50% formamide, 2 x SSC, 10mM dithiothreitol for one hour (Philpott *et al.*1987) followed by 0.1x SSC for 2 x 15 minutes at room temperature. Finally, the slides were dehydrated through a series of ethyl alcohols, air dried, dipped in Kodak

NTB-2 emulsion and stored at 4°C. After 10 days exposure, the slides were developed, counterstained with haematoxylin and photographed.

4.3 Results

Marmoset ovary contains a variety of different cell types such as granulosa cells, theca cells, primordial follicles, secondary, growing and antral follicle types and corpus luteum. Whether ZP transcripts are to be found in oocytes or whether they may also be found in granulosa cells is a controversial issue. The selective detection of ZP3 transcripts by *in situ* hybridisation has enabled us to investigate individual oocytes and follicular cells and determine both the cellular location of the ZP3 transcript and the way in which these signals changed with the stage of oocyte development. Because of the difficulty in using longer length RNA probes in *in situ* hybridisation techniques, a small DNA probe was made using the PCR technique (Figure 4.1). For this purpose marmoset exon 8 was successfully subcloned into the plasmid BluscriptSK- (Figure 4.2). Using plasmid BluscriptSK- containing the marmoset ZP3 exon 8, and T3 and T7 promotors, riboprobes were made in both the sense and antisense orientations. Marmoset ovaries, stored at -80°C were used and 10μm sections were cut using a Cryostat and were hybridized with the sense and antisense probe. The RNA transcripts of marZP3 exon 8 were found hybridize strongly only with oocytes and not with granulosa or corona cells, confirming previous reports in other species (Figure 4.3) (Philpott *et al.* 1987; Lira *et al.* 1990).

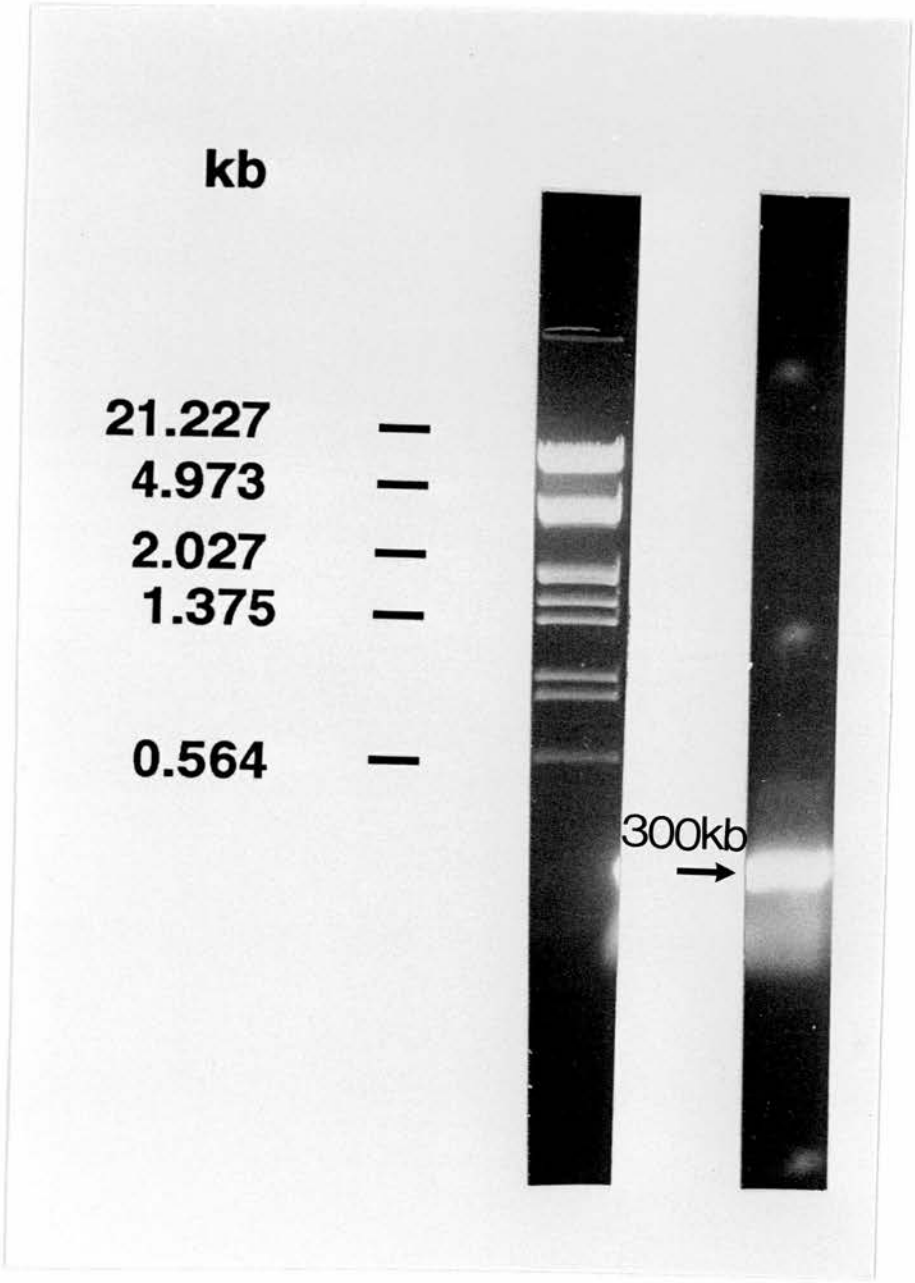


Figure 4.1 Electrophoresis in 0.8% agarose gel showing PCR product of mar ZP3 exon 8. Lane 1, DNA size markers, an *EcoRI* and *Hind III* digest of lamda DNA; Lane 2, PCR fragment of mar ZP3 exon 8, which is indicated by an arrow. The gel was run at 80V for 1-2 hour in 0.5 x TBE.

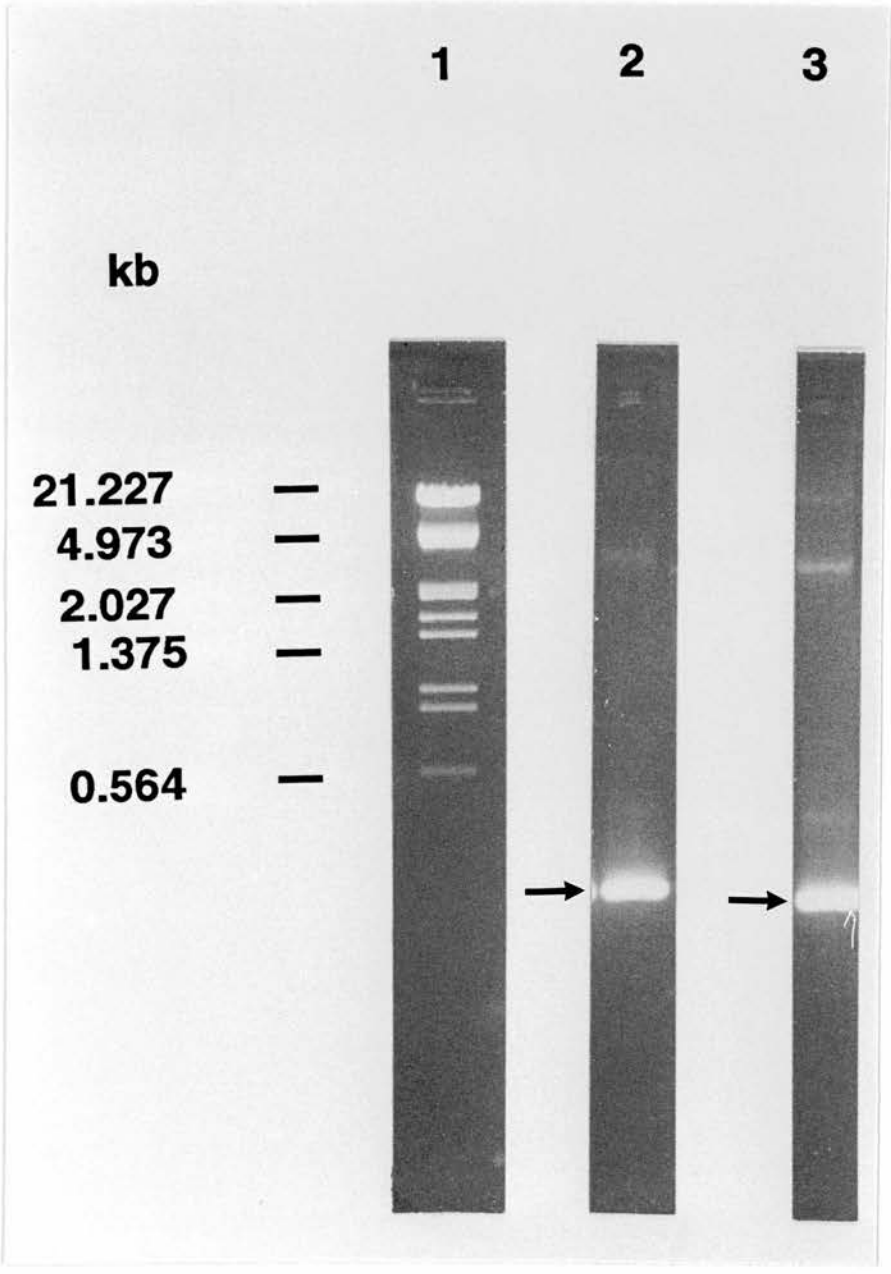


Figure 4.2 Electrophoresis in 0.8% agarose gel showing mar ZP3 exon 8 PCR product. Lane 1, DNA size markers, an *EcoRI* and *Hind III* digest of lamda DNA; Lane 2, Positive clones of mar ZP3 exon-8 in plasmid BluscriptSK- were identified by PCR (fragment of mar ZP3 exon 8 indicated by an arrow); Lane 3, same as lane 2. The gel was run at 80V for 1-2 hour in 0.5 x TBE.

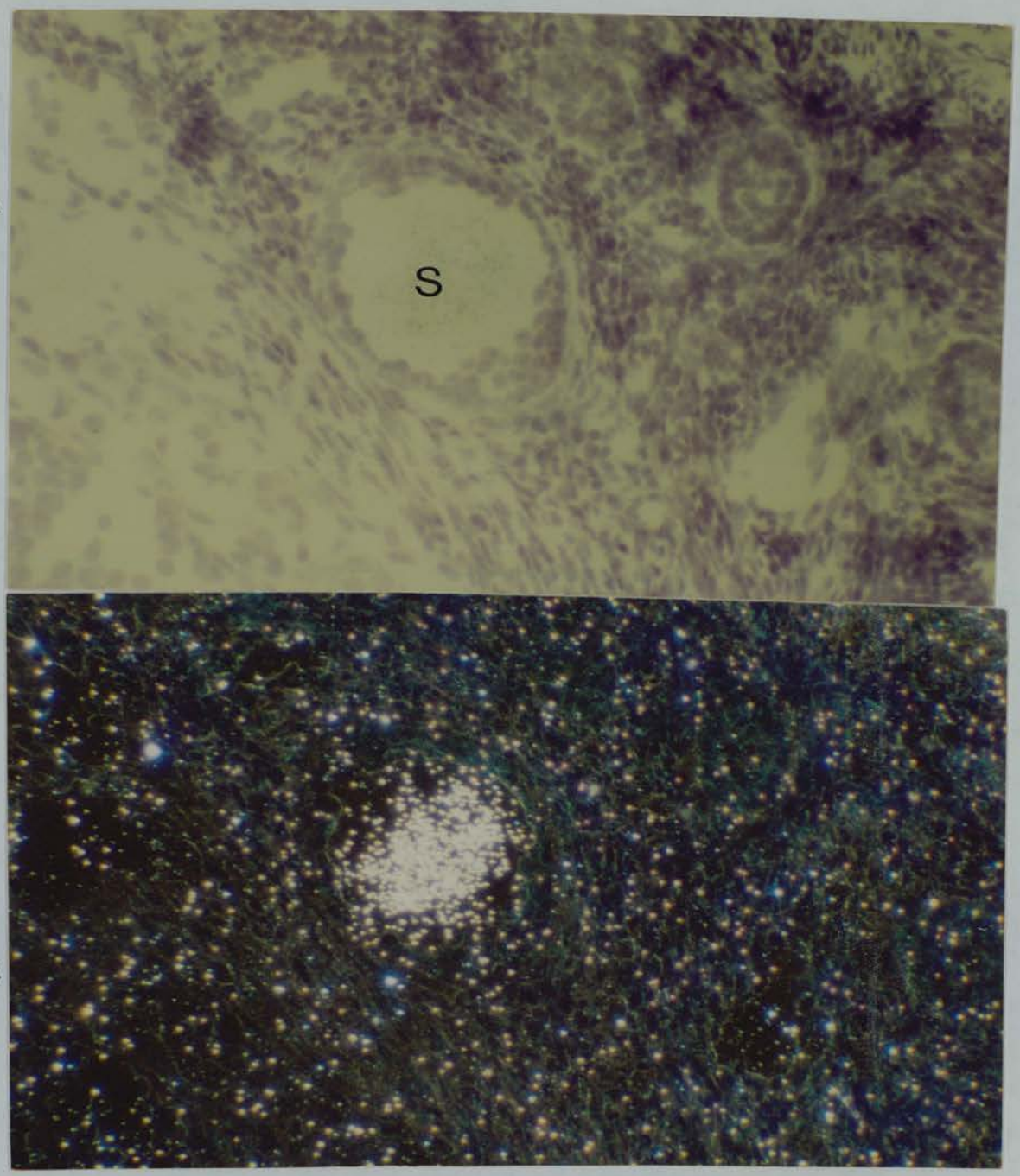


Figure 4.3 *In situ* hybridization of marmoset ovarian tissue. [^{35}S]UTP-labelled antisense RNA transcripts derived from marmoset exon 8 DNA. (A) Haematoxylin stained section after hybridization with antisense RNA transcripts. (B) Darkfield of (A). The RNA transcripts of marZP3 exon 8 strongly hybridized only with secondary follicle (S) and does not involve granulosa or corona cells of the ovary. (100 x)

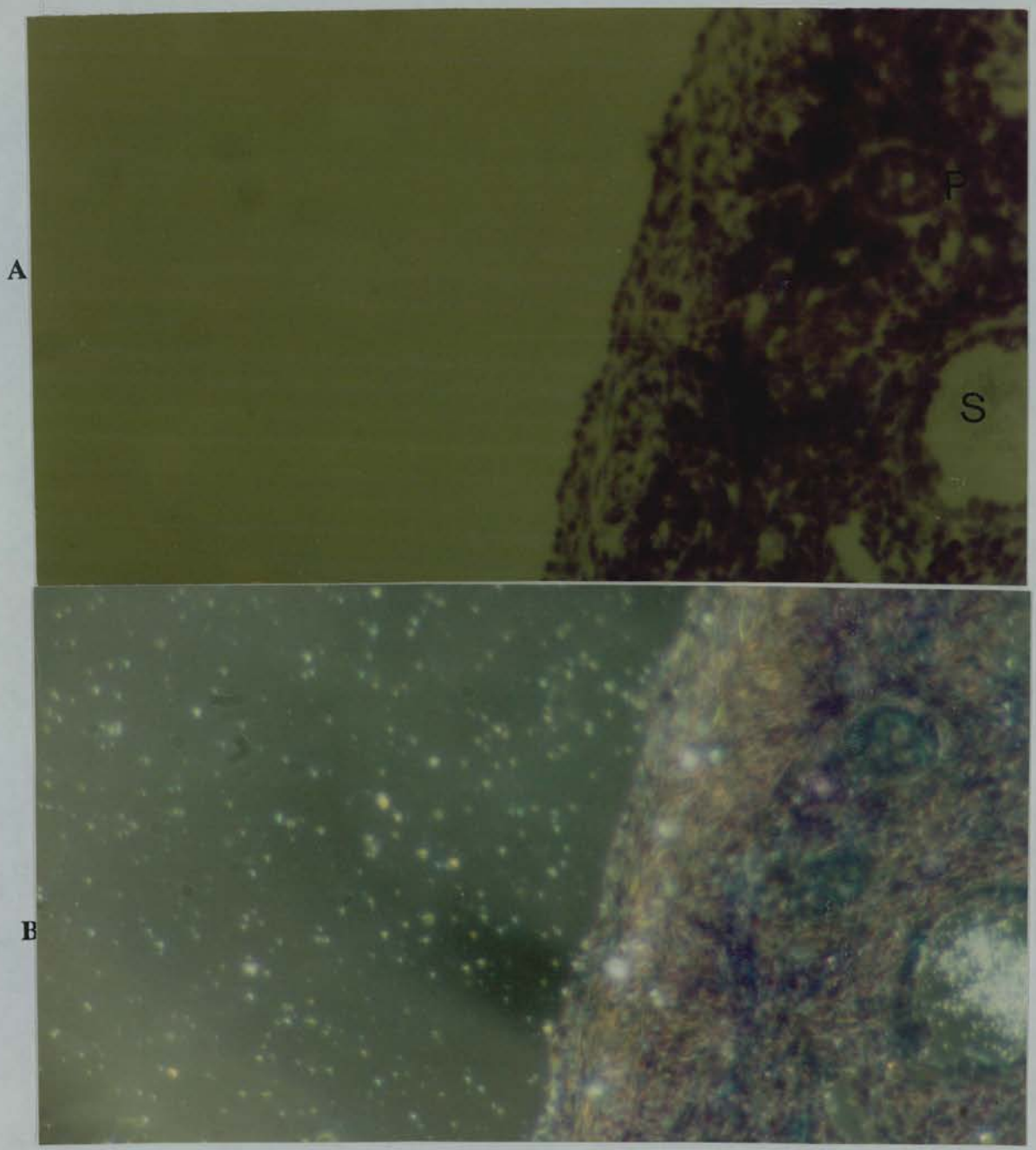


Figure 4.4 *In situ* hybridization of marmoset ovarian tissue. [^{35}S]UTP-labelled antisense RNA transcripts derived from marmoset exon 8 DNA. (A) Haematoxylin stained section after hybridization with antisense RNA transcripts. (B) Darkfield of (A). No ZP3 transcripts were detected in the primordial follicles (P), but ZP3 transcripts could be detected in secondary follicles (S). (62 5 x)

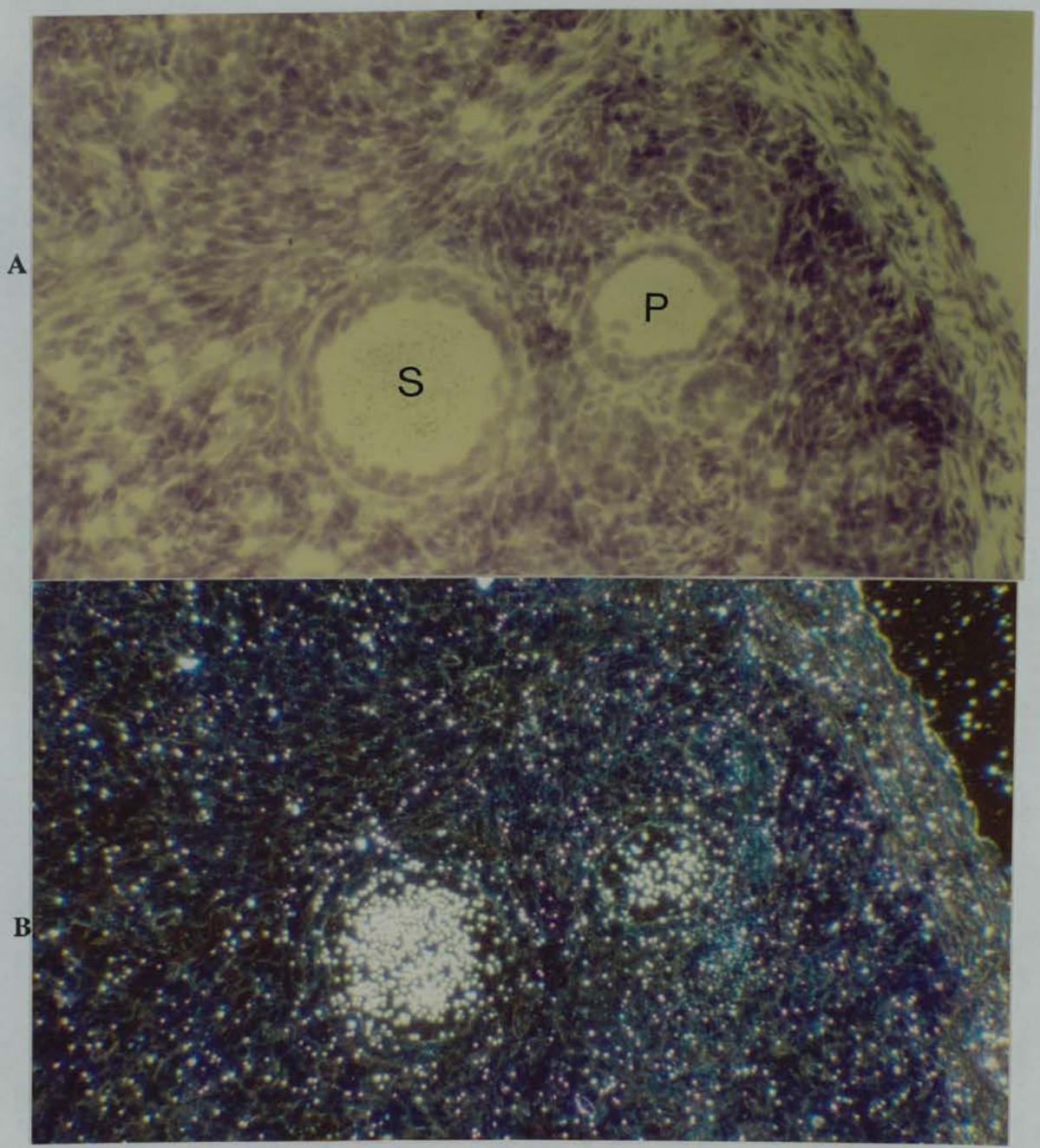


Figure 4.5 *In situ* hybridization of marmoset ovarian tissue. [^{35}S]UTP-labelled antisense RNA transcripts derived from marmoset exon 8 DNA. (A) Haematoxylin stained section after hybridization with antisense RNA transcripts. (B) Darkfield of (A). The ZP3 transcripts are first seen in primary follicles (P) and the intensity of ZP3 transcripts signal increases to a maximum in secondary follicles (S). (100x)

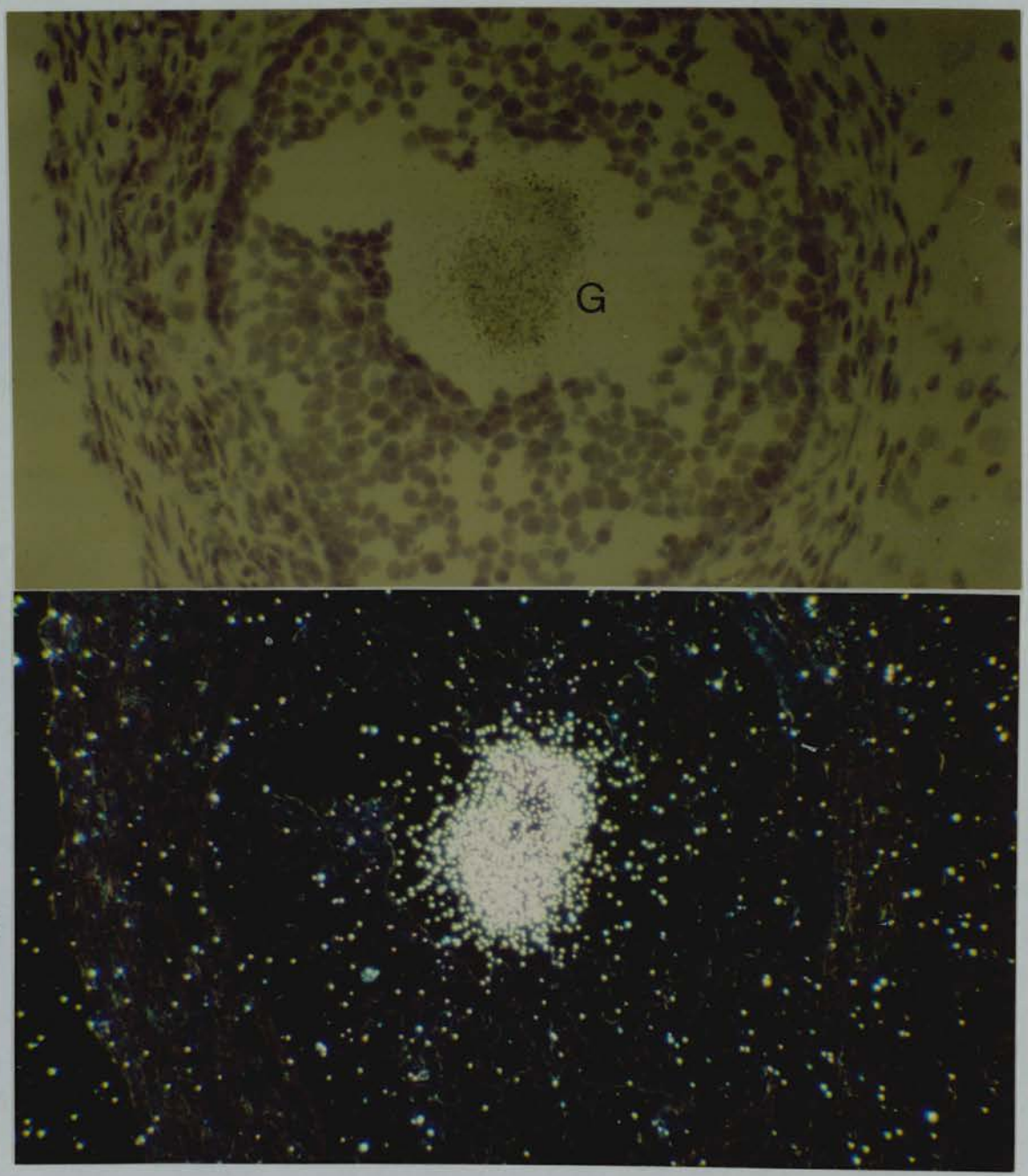


Figure 4.6 *In situ* hybridization of marmoset ovarian tissue. [^{35}S]UTP-labelled antisense RNA transcripts derived from marmoset exon 8 DNA. (A) Haematoxylin stained section after hybridization with antisense RNA transcripts. (B) Darkfield of (A). The ZP3 transcripts could be detected in growing follicles (G) more abundantly than in any other types of oocytes. (100 x)

Since all the *in situ* experiments were done under identical conditions, it is possible to compare the quantity of ZP3 transcripts observed in individual oocytes during oogenesis. No ZP3 transcripts were detected in primordial follicles, but ZP3 transcripts could be detected in primary follicles (Figure 4.4.). The ZP3 transcripts are first seen in primary follicles and the intensity of the ZP3-transcript signals increasesd to a maximum level in secondary follicles (Figure 4.5). The ZP3 transcripts could also be readily detected in the rapidly growing tertiary follicles (Figure 4.6).

To determine whether this expression could be localised to other cells, granulosa and thecal cells of the ovary were examined for the presence of ZP3 transcripts, but no specific signals were present. ZP3 transcripts signals could not be detected in the corpus luteum. Control hybridization experiments involving the sense probe did not give a positive signal with any cells in the ovarian sections.

4.4 Discussion

In situ hybridization was employed to investigate the expression of ZP3 transcripts during oogenesis. The evidence suggests that the ZP3 gene is transcribed exclusively in growing oocytes and not in the surrounding granulosa cells (Liang *et al.*1990; Philpott *et al.*1987). The site of ZP protein synthesis has remained controversial for some time. Earlier studies have pointed out that granulosa cells, which surround the oocytes during folliculogenesis, also produced ZP proteins. The evidence was based upon morphological studies in rodents (Chiquoine, 1960; Hadek, 1965) or on immunoperoxidase staining using an antizona polyclonal antibody in rabbits (Wolgemuth *et al.*1984). The immunostaining appeared to indicate the presence of ZP proteins within the cytoplasm of the inner layer of follicular cells. More recent studies in the rabbit have employed a cDNA encoding for a full-length ZP protein with a

calculated molecular weight of 57,185 (rec55) and a specific antibody to the corresponding native rabbit ZP proteins (R55) (Lee and Dunbar, 1993). Immunohistochemical analysis of ovaries demonstrated that R55 was localized in the follicles of animals 2 weeks and older. R55 was localized in the oocytes of primordial follicles and the oocytes and granulosa cells of primary follicles. Northern blot analysis with the cDNA probe rec55 revealed that cultured granulosa cells contained a mRNA for R55 and that there was stage-specific expression of the R55 mRNA in developing rabbit ovaries (Lee & Dunbar, 1993).

Radiolabelled antibodies or immunofluorescence techniques have also been extensively used to monitor the distribution of monoclonal ZP antigens and, in all cases, the reactivity was found to be directed towards the ZP and not to any other cells of the ovary (Dean & East, 1986; East *et al.*1984a; East & Dean, 1984). In the mouse, these reagents have clearly suggested that the oocyte is the exclusive source of ZP proteins, particularly ZP3. Furthermore, biosynthetic studies involving mouse ZP in an *in vitro* culture system have clearly revealed that the ZP proteins are synthesized in growing oocytes (Shimizu *et al.*1983; Bleil & Wassarman, 1980c). *Cis*-acting sequences located in the mouse ZP3 gene 5' flanking region, together with oocyte-specific proteins, apparently are responsible for the pattern of expression during development. It has been demonstrated that either 6.5 kb or 0.47 kb of the mouse ZP3 gene 5'-flanking sequence can target expression of the firefly luciferase gene exclusively to growing oocytes in transgenic mice (Lira *et al.*1990; Schickler *et al.*1992).

During the oocyte growth phase, each mouse oocyte was found to produce >1 ng of ZP3 for assembly into the ZP (Wassarman, 1988b). It has been reported that mouse oocytes synthesise a significant store of RNA during oogenesis and fully grown oocytes contain 90 pg of poly(A^+) RNA (Bachvarova, 1985; Brower *et al.*1981).

However, fifty percent of poly(A^+) is lost during meiotic maturation and fertilization such that two-cell embryos contain less than 10% of the original maternal poly(A^+) (Levey *et al.*1978; Piko & Clegg, 1982). The level of ZP3 mRNA generation declines dramatically (approximately 98%) during ovulation, from approximately 240,000 copies per oocyte to approximately 5000 copies per ovulated egg. ZP3 mRNA is undetectable in fertilised eggs (less than 1000 copies per fertilised egg), and remains undetectable (less than 1000 copies per embryo) throughout the preimplantation stages of development (Roller *et al.*1989). The results from this study also suggest a similar pattern, where, in the primordial follicle, no ZP3 transcripts can be detected, but in secondary and growing follicles the ZP3 transcripts signals are abundant. It has been observed that in ovulated eggs, that the size of the ZP3 mRNA is approximately 200 nt shorter than it is in the growing oocytes. Since the poly(A) tail length of ZP3 is 200 nt this has led to the notion that the zona mRNAs, like other maternal messages, undergo deadenylation and degradation during meiotic maturation and ovulation. Though the amount of ZP3 mRNA transcripts decline dramatically at the time of ovulation, the ZP itself persists as an extracellular matrix up to the early blastocyst stage and because of its stable configuration serves an important function up to the time of implantation (Wassarman, 1988b). The fact that the expression of the ZP3 genes is restricted to the growth phase of oogenesis in the marmoset suggests that this molecule could be used as a marker for investigating the molecular mechanisms responsible for gene activation in the oocyte.

4.5 Summary

The mar ZP3 transcripts hybridized strongly to oocytes and not to the surrounding granulosa cells. Primordial follicles did not contain detectable amounts of marmoset ZP3 transcripts. The mar ZP3 transcripts were detected in primary oocytes and the accumulation of ZP3 message continued in secondary and tertiary follicles. This study has clarified the controversy over the site of ZP3 synthesis as least as far as the marmoset is concerned. The oocyte-specific accumulation of ZP3 transcripts could be used as a marker to study other factors involved in the activation of the oocyte following the recruitment into the growing follicle pool

5 Cloning and Expression of Marmoset ZP3

5.1 Introduction

The development of techniques to manipulate DNA *in vitro* in the 1970s has had a major impact on the production of key proteins, of limited natural availability, in abundant quantity. The expectation that foreign DNA should be expressed in unrelated organisms is based on the assumption that the genetic code is universal. Unfortunately, when DNA from higher eukaryotes was tried in such systems, expression of the foreign genes was not obtained. In order for eukaryotic genes to be expressed in *E. coli* they must be modified in such a way that their organisation resembles prokaryotic genes. In order for a eukaryotic gene to be transcribed within a prokaryotic cell, it is necessary to place the eukaryotic gene downstream of the prokaryotic promotor. Most plasmid expression vectors utilise either the promotor of the *lac* or of the *trp* operons from *E. coli*. Many of the vectors contain enough of the *lacZ* gene to encode a peptide of ß-galactosidase. The *lac* operon is negatively regulated by the *lac* repressor, and can be derepressed by addition of an inducer, such as isopropyl ß-D-thiogalactoside (IPTG). Genes can be directed to express their products either in the cytoplasm or, by incorporating a leader sequence before the coding sequence, the proteins may be secreted through the cell membrane. The level of expression in the cytoplasm is greater than the secreted protein. However, many of the polypeptide products located in the cytoplasm are insoluble and aggregated and specific solublization procedures are needed (Marston, 1988). Problems associated with the instability of the recombinant proteins due to host proteases were overcome

by adopting fusion protein expression strategies which also gave good translation initiation. The ß-galactosidase system has been used extensively as a fusion protein expression system. Recently this fusion protein expression strategy has been improved further by including a signal peptide which can be cleaved with specific proteases (Moks *et al.*1987). Furthermore, the protein of interest can be selectively attached to a fusion protein which can bind to a specific ligand which can then be used on the basis for an affinity column purification procedures (Guan *et al.*1987).

ZP from mammalian species has been difficult to isolate in large quantities either due to the difficulty in collecting it from the ovary or because of the paucity of the ovarian material itself (human, primate ovary). Producing ZP3 by genetic engineering is an alternative approach. Mouse ZP3 and hamster ZP3 genomic DNA have been transfected into the embryonal carcinoma cell lines of mouse origin and expressed successfully (Kinloch *et al.*1991). The recombinant mouse ZP3 produced was biologically active, and could bind to mouse sperm and induce the acrosome reaction. Moreover, the amount of recombinant ZP3 generated was large enough to perform biochemical analyses. Mouse ZP3 cDNA transfected into rodent and primate cell lines also generated biologically active ZP3 with differing molecular mass Mr=60,000-70,000; (Beebe *et al.*1992). Schwoebel *et al* (1992) have expressed rabbit ZP2 and ZP3 cDNAs in bacteria and the recombinant ZP proteins produced (rec55) were immunogenic in cynomologus monkeys when conjugated to either protein A or keyhole limpet haemocyanin. In this context, production of recombinant marmoset ZP3 would be very useful to use as an antigen in contraceptive vaccine development. A commercially available protein expression and purification system has been used to clone the marmoset ZP3 cDNA. Using this system (pMAL™ vector) the gene was inserted downstream from the *malE* gene, which encodes a maltose binding protein

(MBP), and results in the expression of an MBP fusion protein achieved (Guan *et al.*1987).

5.2 Materials and Methods

5.2.1 Ligation of DNA Fragment with Low Melting Agarose Gel

DNA, digested with particular restriction enzymes, was run on a 2% Nusieve (Flowgen; Nusieve GTG agarose; FMC Bioproducts, High Wycombe, Bucks, UK) agarose gel and then the fragment was sliced out of the gel for further ligation. The agarose slice containing the DNA was melted at 68°C for 10 minutes and then placed at 37°C in a water bath until the addition of the ligation reaction mix. Water was added, approximately 3 times the volume of the melted agarose used for the ligation reaction, but not exceeding a volume of 30µl. The 10x ligase buffer volume was 1/10th of the volume of the ligation reaction mixture and 1 unit of T4 DNA ligase per 10µl of the ligation mixture was added. The vector was added to obtain a molar ratio of insert to vector of 5:1 (Dumais & Nochumson, 1987).

5.2.2 Subcloning in the pMAL^{TM}-c Vector

Marmoset ZP3, exon 1-6 in the PCR 1000 vector, was digested and opened with Sac1 and Sal1 restriction enzymes. The digested DNA was run on a 2% Nusieve agarose gel and the digested fragment was sliced out for further ligation. Similarly, the marmoset ZP3 exon 4-8 in the PCR 1000 vector was digested with Sac1 and Sal1 restriction enzymes and run on a Nusieve agarose gel (Flowgen; Nusieve GTG agarose; FMC Bioproducts, High Wycombe, Bucks, UK) and the fragment was sliced from the gel for further ligation. The two agarose gel DNA fragments were melted at 68°C for 10 minutes and then kept at 37°C before adding to the ligation mixture. The

ligation mixture contained: agarose gel of marmoset ZP3 exon 1-6 in PCR 1000 vector opened with Sac1 and Sal1 restriction enzymes (2µl), agarose gel of marmoset ZP3 exon 4-8 fragment digested Sac1 and Sal1 from PCR 1000 vector agarose gel (10µl), 10 x ligation buffer (5µl), T4 DNA ligase (1µl, 4 Units) and water (32µl). The ligation reaction was carried out at room temperature overnight. The following day the ligation mix was once again melted at 68°C for 5 minutes and then electrotransformed in XL1-B cells. White colonies of putative positive clones were grown in LB media and mini-plasmids were prepared using Magic Mini-Prep columns (Promega, Southampton, UK). The plasmid preparations were digested with Kpn1 restriction enzyme and verified on agarose gels for positive plasmids carrying the marmoset ZP3 cDNAs.

The PCR 1000 vector carrying the marZP3 was digested with EcoR1 restriction enzyme and run on 2% Nusieve agarose gel (Flowgen, FMC Bioproducts, High Wycombe, Bucks, UK) and the 1.3kb fragment sliced out of the gel for the ligation reaction. The pMALc^{TM}-c vector (New England Bio lab, Herts, England) was digested with EcoR1 restriction enzyme and then the vector was dephosphorylated with calf intestine alkaline phosphatase. The EcoR1 cut plasmid DNA was mixed with 5µl, 10 x calf intestine alkaline phosphate buffer, calf intestine alkaline phosphatase (4 units) and water to make a total volume of 50µl. The reaction was carried out at 37°C for 60 minutes. The reaction was stopped with 1µl of 0.5M EDTA and then the calf intestine alkaline phosphatase activity was heat destroyed at 75°C for 10 minutes. The dephosphorylated plasmid DNA was phenol:chloroform extracted twice and then ethanol precipitated.

The Nusieve agarose slice containing the marZP3 fragment, digested with EcoR1, was melted at 68°C for the ligation reaction as described (Section 5.2.1). The dephosphorylated pMALc^{TM} vector was added to the ligation mixture and the reaction

was carried out overnight at room temperature. The following day the ligation product was electrotransformed with JM105 cells. White colonies were grown in LB medium and the purified mini-plasmid was digested with restriction enzyme Pst1 to determine whether marZP3 was present in the right orientation.

5.2.3 Analysis of Recombinant mar ZP3 Protein

Plasmid carrying mar ZP3 was grown in 5ml LB medium with ampicillin (50µg/ml) to about 2 x 10^8/ml. A 1 ml sample was spun in a microcentrifuge for 2 minutes and the pellet was resuspended with 50µl of protein gel SDS-PAGE sample buffer. Inducer, IPTG, was added to the remaining culture to a final concentration of 1mM and the cells were grown for another 2-3 hours. A 0.5 ml sample was centrifuged for 2 minutes and the pellet was resuspended with 100µl of SDS-PAGE sample buffer. The remainder of the cells were centrifuged and resuspended with 2ml of column buffer (20mM Tris-HCl; pH 7.4; 100mM NaCl; 1mM EDTA). The sample was frozen at -20°C overnight and thawed in cold water the following day and treated with 1mg/ml lysozyme for 30 minutes on ice. This material was then sonicated using a MSE Soniprep 150 for 3 x 20 seconds, followed by centrifugation at 9000g for 20 minutes. The supernatant (crude extract 1) was saved and 5µl was treated with 5µl SDS-PAGE sample buffer. The pellet was resuspended with 2 ml of column buffer to create a suspension of the insoluble matter (crude extract 2). This 'crude extract 2' was also treated with 5µl SDS-PAGE sample buffer. All the samples were boiled for 5 minutes and 15µl of each sample was used for 10% SDS-PAGE analysis.

5.2.4 Electrophoresis

One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was performed according to the method of Laemmli (1970) using a Protean

electrophoresis cell (Hoeffer Scientific Instruments, Newcastle, UK.). The stacking gel was composed of 3.6% acrylamide, pH 6.8 and the resolving gel contained 10% acrylamide, pH 8.8. Samples from different bacterial lysates containing recombinant marmoset ZP3 were run under non-reducing conditions. Molecular weight standards (Rainbow markers, Amersham International, UK) were also run. Electrophoresis was performed at a constant current of 20mA for 2 hours through the resolving gel using Tris, glycine, 0.1% SDS as electrode buffer. Some gels were stained with Coomassie Brilliant blue G-250 whereas others were used for Western blotting as described in section 5.2.5. After electrophoresis the gels were stained with 0.5% Coomassie Brilliant blue in 25% isopropanol with 10% acetic acid for 2-3 hours, destained with 25% isopropanol and 10% acetic acid for 1-2 hours and replaced with 10% isopropanol and acetic acid overnight.

5.2.5 Western Blot

The SDS-PAGE gels were soaked in Western blot transfer buffer for 10 minutes along with nitrocellulose membranes (Amersham Inernational, UK). Using a BioRad transfer blot apparatus the proteins were transferred under a constant current of 0.22A for 4 hours using 25mM Tris-HCl buffer, pH 8.3, 192mM glycine, 20% methanol. The nitrocellulose membranes were blocked with 5% milkpowder (Marvel, Premier Brands UK. Ltd., Stafford, UK.) containing 0.1% Tween 20 for 30 minutes followed by a brief wash with PBS. The nitrocellulose membrane was incubated with 1/1000 anti-human ZP3 peptide 341-360 antibody overnight at room temperature (The peptide 341-360 antibody was a gift from Organon Pharmaceuticals, Oss, Netherlands). The next day the membrane was twice washed with 0.1M PBS, pH 7.2, containing 0.1% Tween 20 for 15 minutes. Anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) was employed as the second antibody for 1 hour. Reactivity was visualised

by autoradiography using an enhanced chemiluminescence detection system (Amersham International plc, Bucks, England).

5.3 Results

A schematic diagram of the appropriately digested plasmids from PCR 1000 vector containing marZP3 exon 1-6 and 4-8 cloned into the pMAL™-c vector is presented in Figure 5.1. The marZP3 exon 1-6 fragment was restriction enzyme digested with SalI and SacI enzymes to create an opening at position 731nt in the mar ZP3 and the SacI polylinker site of the PCR 1000 Vector (Figure 5.2), to insert the rest of the marZP3 4-8 fragment digested with the same restriction enzymes (SacI and SalI). In this way a full length mar ZP3 construct was made in PCR 1000 vector (Figure 5.3) which could be easily subcloned in the pMAL™-c vector (Figure 5.4).

A full-length marZP3 cDNA was subcloned in the pMAL™-c vector, (New England Biolab, Herts, England) downstream of the *malE* gene which encodes maltose binding protein (MBP) and results in the expression of an MBP fusion protein. The strong "tac" promoter and the *malE* translation initiation signal present upstream of the cloned marZP3 gave high-level expression of marZP3. The pMAL™-c vectors also contain the sequence coding for the recognition site of the specific protease factor Xa, which allows MBP to be cleaved from marZP3 after purification.

The marmoset ZP3 cDNA, exhibiting a size of 1.3kb, was cloned at the EcoR1 site downstream from the malE gene, in the pMAL™-c vector which encodes the maltose binding (MBP) fusion protein. The pMAL™-c vector-containing JM105 cells were grown to an A_{600} of 0.5, then induced by addition of IPTG, to stimulate a high level of expression of the marmoset ZP3 fusion protein. The molecular weight of the MBP is 42,000 kD, resulting in the expression of an MBP fusion protein with total

molecular mass of 86,000 as determined by SDS-PAGE (Figure 5.5). The pMAL™-c vector *malE* gene has a deletion of the signal sequence, leading to cytoplasmic expression of the fusion protein. In this site the fusion protein was present in a soluble and stable form as shown by SDS-PAGE. Analysis of the bacterial fusion proteins by Coomassie blue staining separated by 1D SDS-PAGE is shown in Figure 5.5. Highly expressed fusion protein is visible in the sample (Figure 5.5) which was induced with IPTG, compared to the uninduced control.

To determine whether or not the induced cells synthesized marZP3 fusion protein, cell lysates were prepared and analysed on immunoblots as well as Western blots using rabbit sera raised against a synthetic peptide of human ZP3 (peptide sequence of 340-361) (Figure 5.6). This antibody recognizes marZP3 fusion protein in the bacterial cell lysate that was induced with IPTG, with the expected size 86,000 kD. By control, in the uninduced bacterial cell lysate the antibody could not detect any peptide of similar size. The antibody also detected a number of bands both in the uninduced and induced bacterial cell lysates. A mouse monoclonal antibody MCA7 raised against human ZP3 also recognises marZP3 fusion protein in the bacterial cell lysate that is induced with IPTG (Figure 5.7).

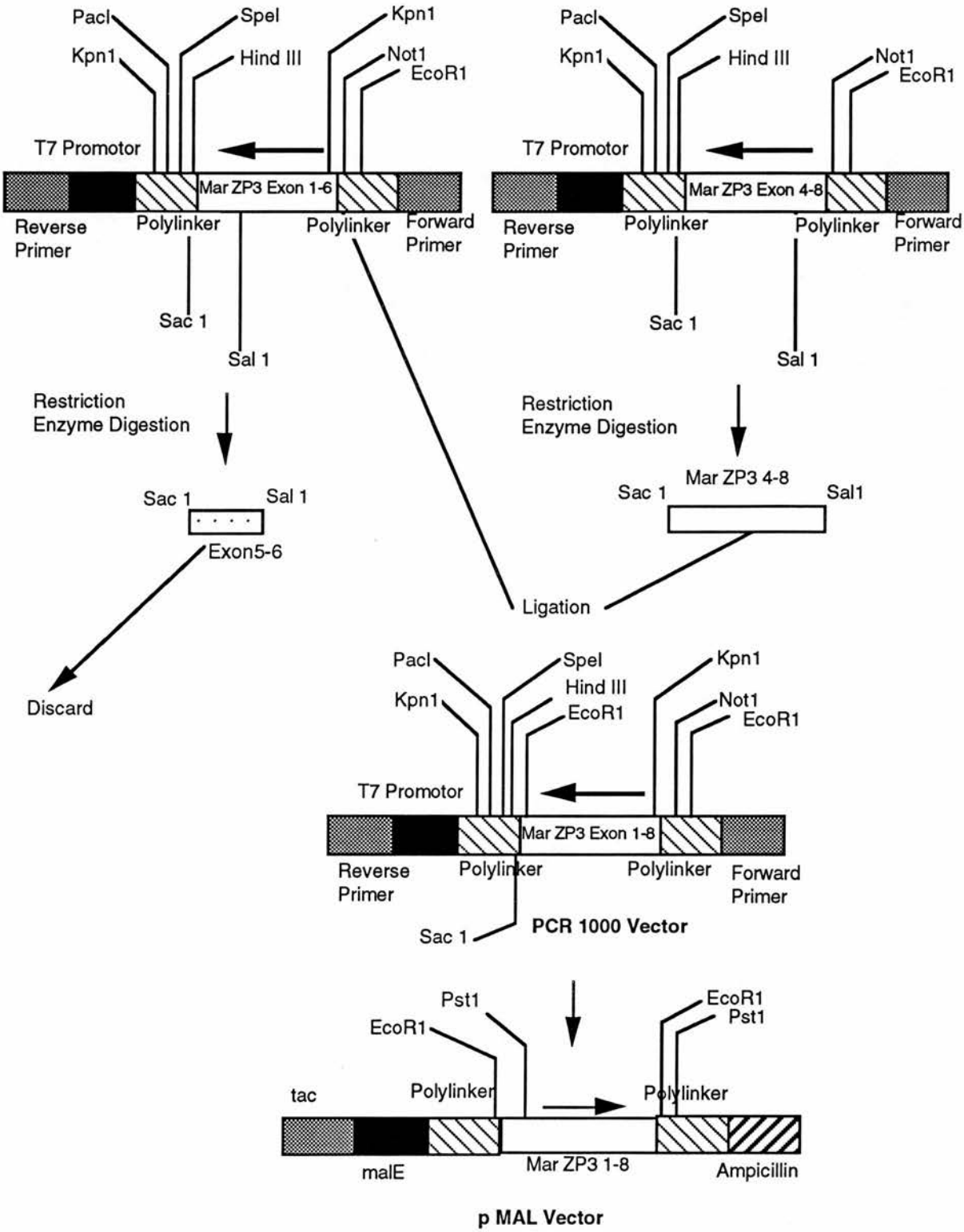


Figure 5.1 Diagrammatic representation of marmoset exon 1-8 subcloning in the pMALTM-c vector.

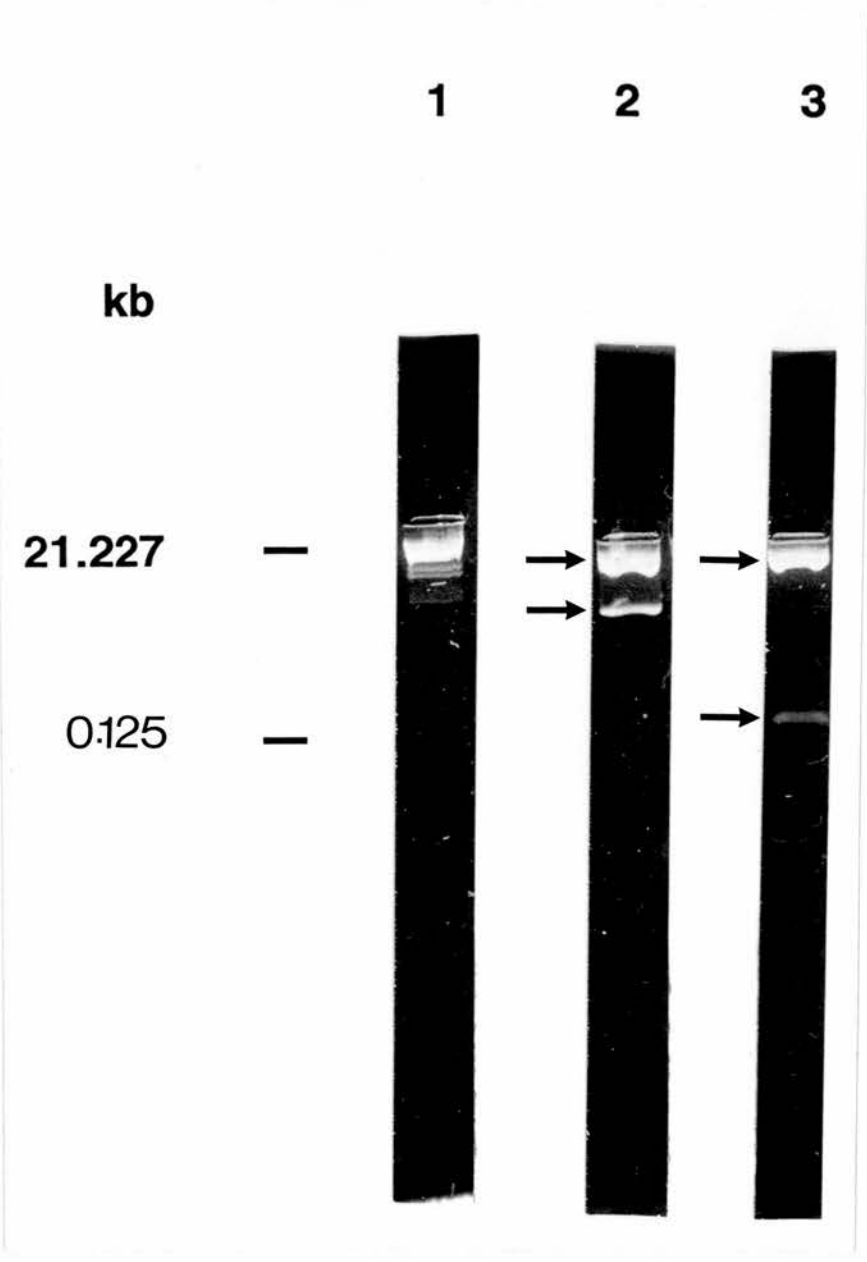


Figure 5.2 Electrophoresis in 2% Nusieve agarose gel. Lane 1, DNA size markers, an *EcoRI* and *Hind III* digest of lamda DNA; Lane 2, PCR 1000 vector containing mar ZP3 exon 4-8 digested with Sac1 and Sal 1 restriction enzyme producing vector band at 3 2kb and release of insert of 600 bp as indicated by arrows; Lane 3, PCR 1000 vector containing mar ZP3 exon 1-6 digested with Sac1 and Sal1 restriction enzyme producing vector and mar ZP3 at 3.7kb and release of insert of 125 bp as indicated by arrows.

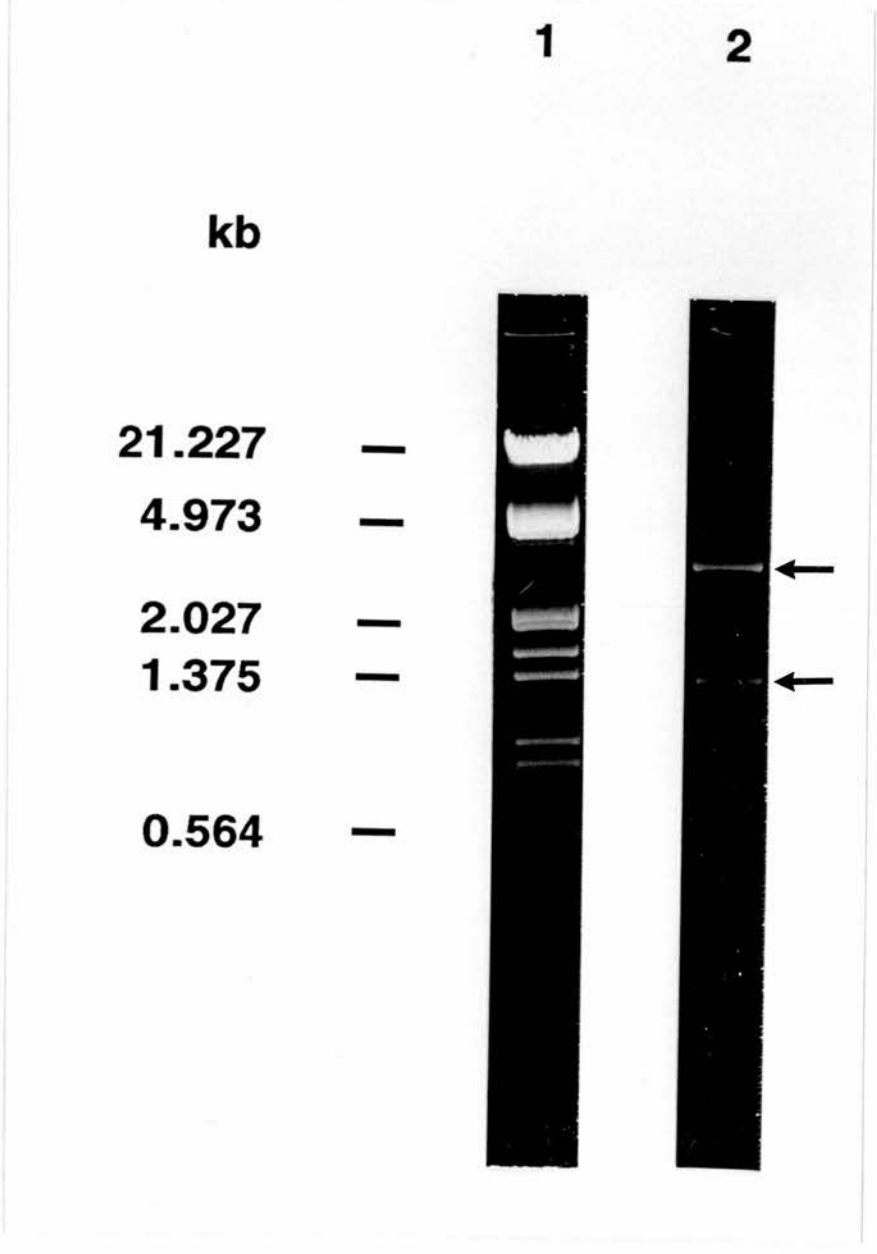


Figure 5.3. Electrophoresis of subcloned mar ZP3 in PCR 1000 vector in an 0.8% agarose gel. Lane 1, DNA size markers, an *EcoRI* and *Hind III* digest of lamda DNA; Lane 2, plasmid preparation were digested with Kpn1 restriction enzyme producing vector band at 3.2kb and release of mar ZP3 insert of 1.2kb.

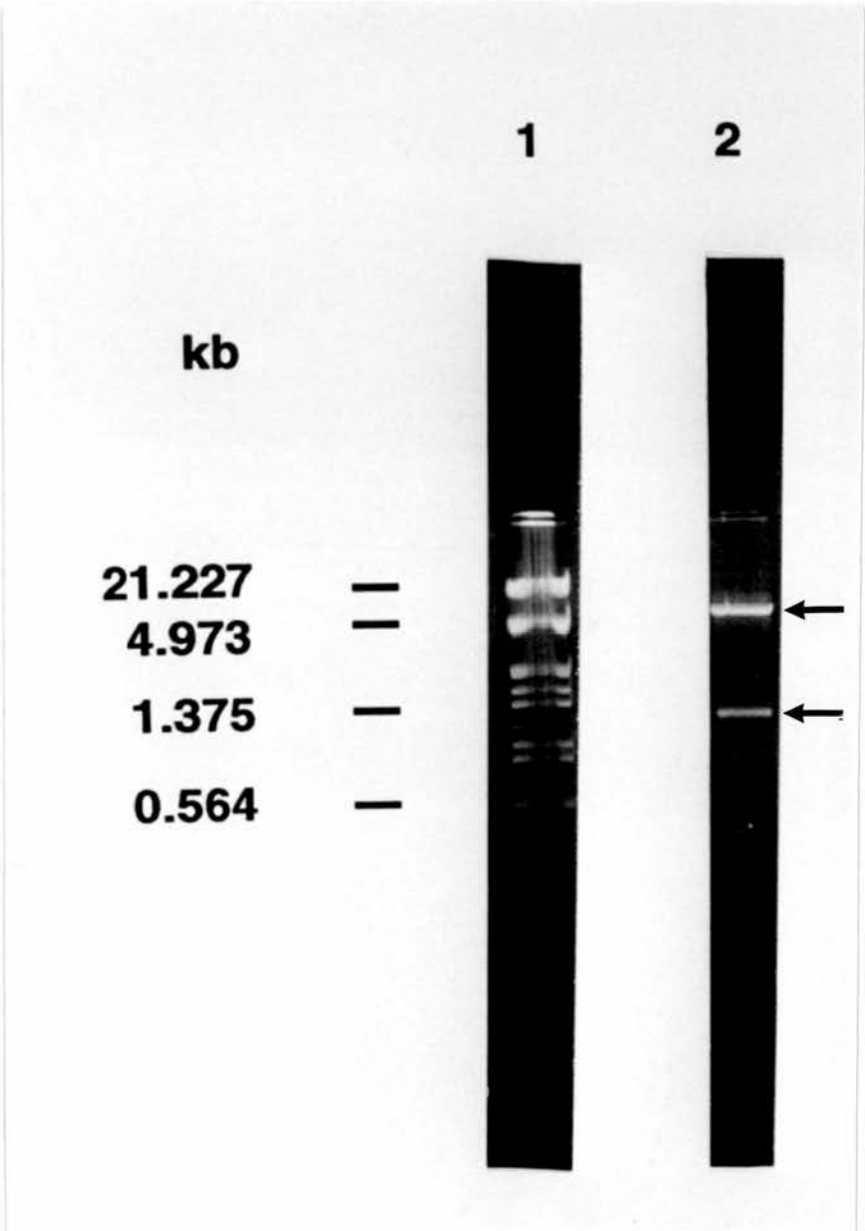


Figure 5.4. Electrophoresis of subcloned mar ZP3 in pMALc™-c vector in an 0.8% agarose gel. Lane 1, DNA size markers, an *EcoRI* and *Hind III* digest of lamda DNA; Lane 2, plasmid preparation were digested with EcoR1 restriction enzyme and verified on agarose gel for positive plasmid carrying the marmoset ZP3 gene. The pMALc™-c vector band at 6.1kb and release of mar ZP3 insert of 1.3 kb as indicated by the arrows.

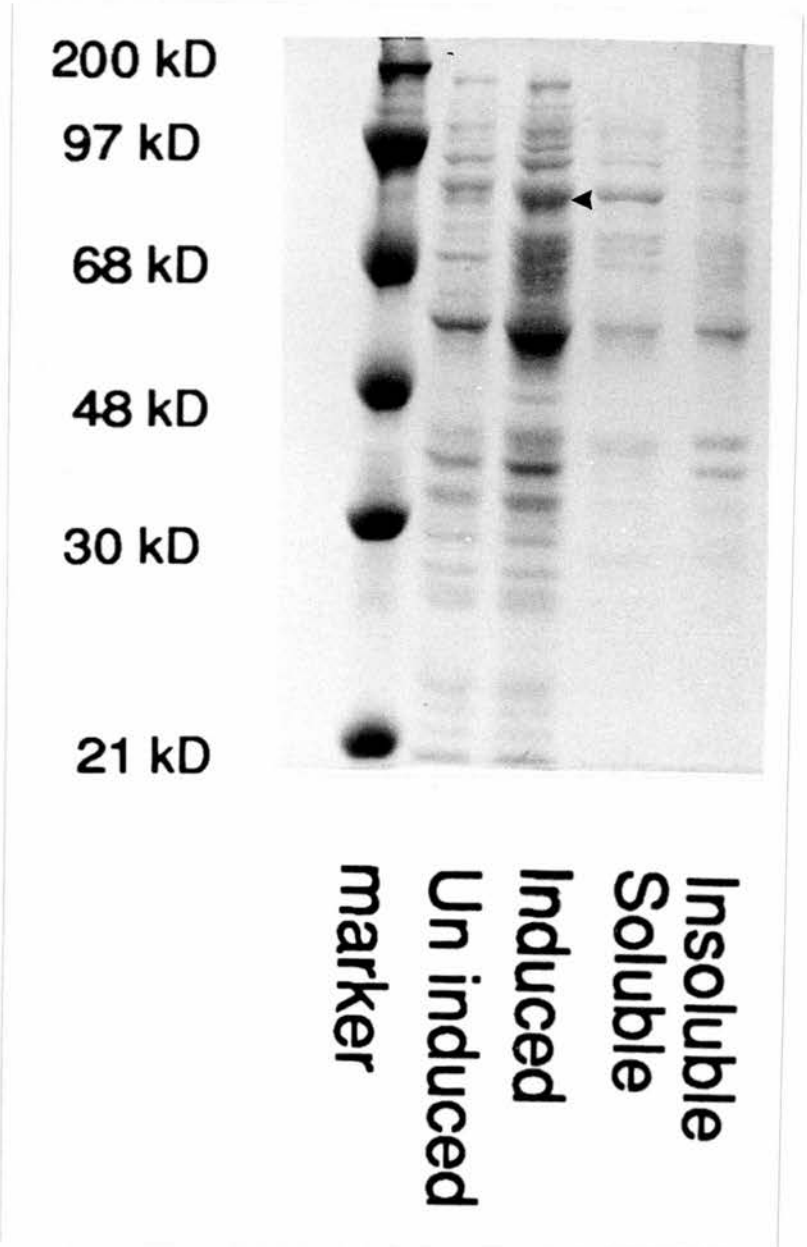


Figure 5.5 Analysis of the bacterial fusion proteins by Coomassie blue staining of proteins separated by 1D SDS-PAGE. Molecular weight markers as indicated on left hand side of the gel. Uninduced lane contained lysed bacterial cell before inducing with IPTG. Induced lane contained lysed bacterial cell after inducing with IPTG. The marmoset ZP3 fusion protein is indicated with an arrow head. Soluble lane contained lysed bacterial cell extract solublized with Tris-HCl buffer, pH 7.4. Insoluble lane contained lysed bacterial cell extract's pellet after solublization with Tris-HCl buffer, pH7.4.

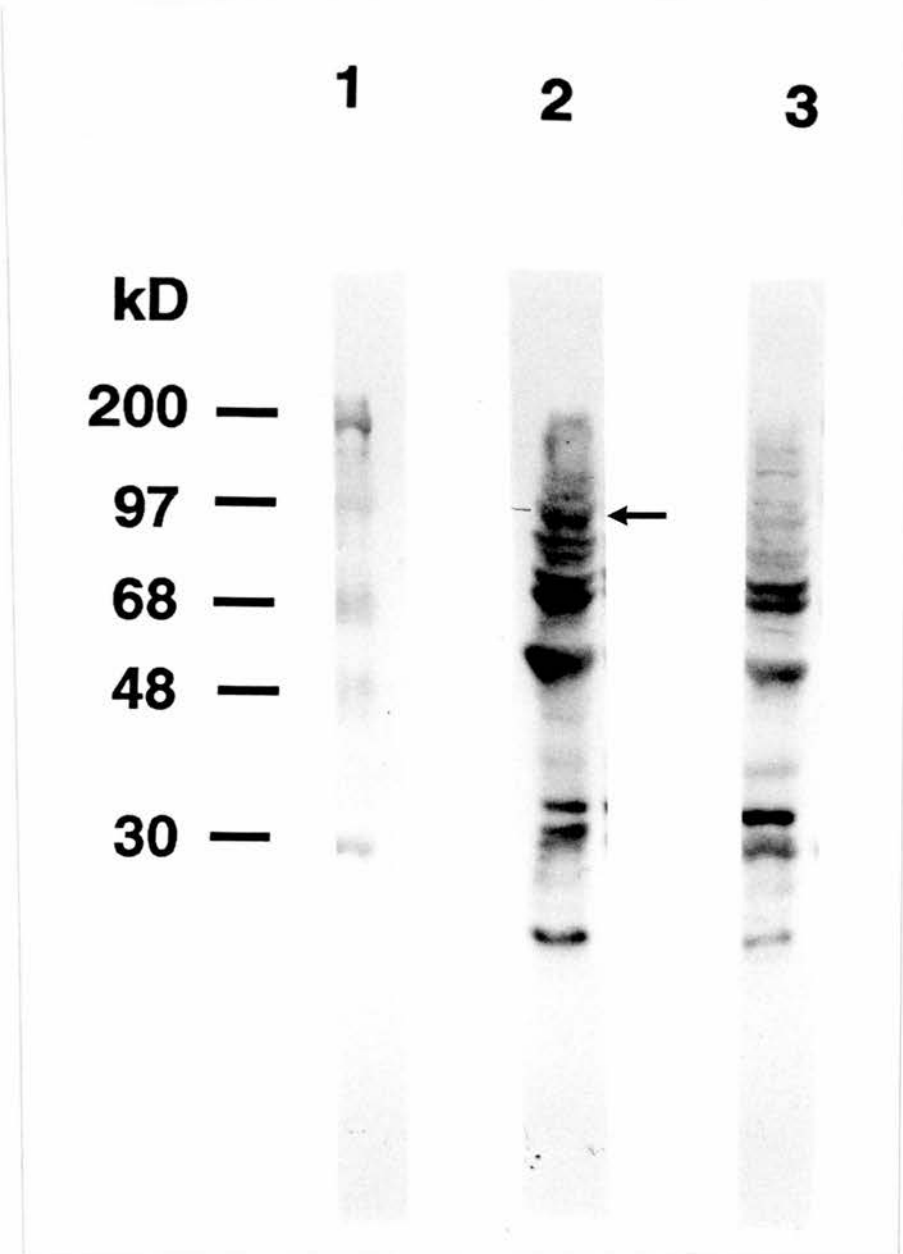


Figure 5.6 Blots of lysed bacterial cell extract containing marmoset ZP3 fusion protein probed with rabbit sera raised against a synthetic peptide of human ZP3 (peptide sequence of 340-361). This antibody recognizes marZP3 fusion protein (lane 2, indicated by an arrow) in the bacterial cell lysate that is induced with IPTG of expected size 86,000 kD protein. In the uninduced bacterial cell lysate (lane 3) the antibody could not detect any peptide of similar size. Molecular weight markers (lane 1) are indicated on left hand side of the gels.

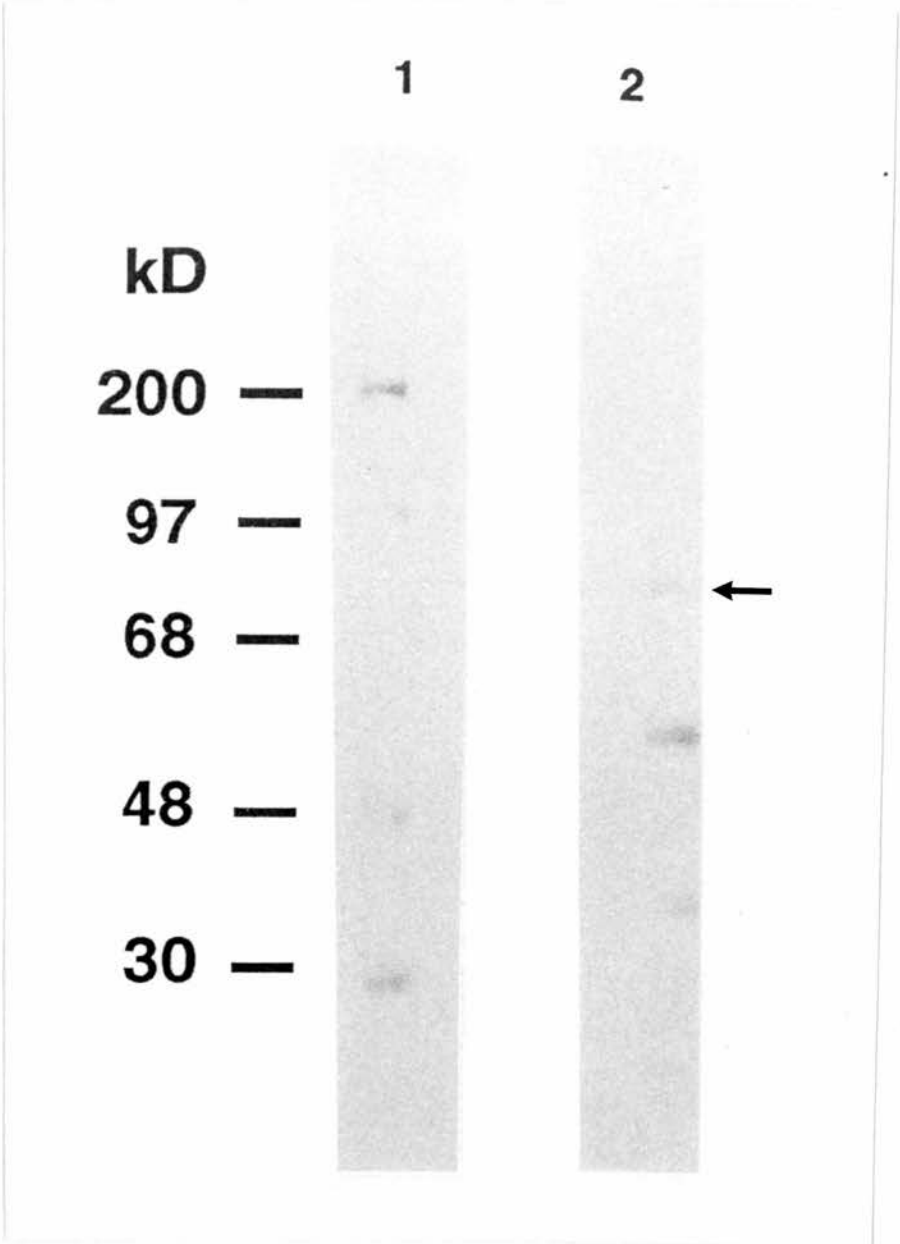


Figure 5.7 Blots of lysed bacterial cell extract containing marmoset ZP3 fusion protein probed with a mouse monoclonal antibody MCA7 raised against human ZP3. This antibody recognizes marZP3 fusion protein (lane 2, indicated by an arrow) in the bacterial cell lysate that is induced with IPTG of expected size 86,000 kD protein. Molecular weight markers (lane 1) are indicated on left hand side of the gels.

5.4 Discussion

The data presented in this study describe a novel bacterial expression system that is capable of producing high levels of a soluble stable marZP3 fusion protein. The marZP3 full length cDNA of 1.3kb was cloned downstream of the *malE* gene resulting in a fusion protein containing marZP3 coupled with the maltose binding protein. Extracts of *E. coli* JM105 contained a prominent polypeptide with molecular mass 86,000 Daltons. The fusion protein produced by this system was in a soluble form in aqueous solutions. The fusion protein produced by this vector was not binding to the amylose resin (affinity purification column) which might be due to change in the MBP confirmation that was masked by mar ZP3 protein. Due to this problem an anion exchange chromatography was used for the fusion protein purification, which is discussed in chapter 6.2.2. Schwoebel *et al* (1992) have produced a rabbit ZP recombinant protein of molecular mass 55-kD (rec55) with cro-ß-galactosidase as the fusion protein. However these fusion proteins were insoluble in the absence of urea and prone to aggregation in aqueous solutions. It has been noted that high level expression of fusion peptides in *E. coli* may result in the expressed products being sequestered into inclusion bodies or protein aggregates (Williams *et al.*1982). Rabbit recombinant ZP protein, (rec55) was expressed in pEX plasmid in these insoluble inclusion bodies (Schwoebel *et al.*1992b). The fact that marZP3 produced with MBP protein as the fusion protein was soluble in aqueous solution might be due to the different type of vector employed. This fusion protein (MBP) might also help to establish the correct 3-dimensional configuration for the protein and the formation of a stable translation initiation complex. Embryo carcinoma cell-derived recombinant mouse ZP3 inhibited mouse sperm-egg binding *in vitro* and also induced mouse spermatozoa to acrosome react, but hamster ZP3 expressed in the same cell line was

not biologically active, suggesting the significance of protein configuration and post translational modification in the expression system employed (Kinloch *et al.*1991). However it should be noted that Beebe *et al* (1992) have shown that the full length mouse ZP3 cDNA construct cloned in cytomegalo virus and transfected into the mouse L-929 cell line produced mouse recombinant ZP3 with molecular wt of 60,000-70,000. Another construct cloned in vaccinia virus promotor and transfected into the green monkey CV-1 cell line also produced recombinant ZP3 of the same size. Both types of recombinant glycoproteins were biologically active since they inhibited sperm binding to the zona pellucida and induced the acrosome reaction despite presumed differences in the glycosylation patterns achieved with these two expression systems.

The marZP3 was analysed by Western blot with antibodies produced against the human ZP3 peptide, 340-361, in rabbits. This antibody recognizes marZP3 fusion protein as shown in Figure 5.5. Furthermore, it suggests that prokaryote-produced marZP3 expresses the appropriate specific antigens, since it is recognized by antibodies. Significantly, the fact that recombinant marZP3 produced with maltose binding protein as a fusion product is still antigenic suggests that the major epitopes on the marZP3 protein are not masked by the maltose binding protein. Moreover, a mouse monoclonal antibody (MCA7) generated against human ZP3 also cross reacted with marZP3 fusion protein, indicating recognition of major marZP3 epitopes in this fusion protein by antibodies raised in different species.

The cross reactivity between the anti-human ZP3 peptide antibody and mar ZP3 is clearly a consequence of the similarity in amino acid sequence between human ZP3 and marZP3 (Thillai-Koothan *et al.*1993). However, the rabbit antibody against human ZP3 peptide 341-360 also cross-reacted with a number of other peptides in the induced and uninduced bacterial cell lysate (Figure 5.6). Nevertheless, it could not detect any peptide of similar size to the fusion protein in the uninduced bacterial cell

extract. The reason for the non-specific cross-reactivity is unclear. However unabsorbed polyclonal antibodies cross reacting with prokaryotic proteins have been noticed with a number of other expression system (Huynh *et al.*1985).

The application of the expression system described in this chapter should result in the supply of sufficient quantities of purified recombinant marZP3 for further studies in the context of contraceptive vaccine development and the fundamental cell biology of the ZP. The ability to generate purified recombinant material is important since the abnormal ovarian cyclicity seen in some ZP immunization studies might have been due to the presence of impurities in the immunogen (Gulyas *et al.*1983). This approach of producing recombinant proteins in *E. coli* eliminates any possible contamination by unwanted ovarian antigens in contraceptive vaccine studies and in this respect represents a considerable advance over the isolation of native protein for immunization purposes.

5.5 Summary

Marmoset ZP3 cDNA, exhibiting a size of 1.3kb, was constructed and cloned downstream from the malE gene, in the pMALc^{TM}-c vector which encodes the maltose binding (MBP) fusion protein. The fusion protein containing mar ZP3 was soluble in aqueous solution and the size of the fusion protein was 86 kD as expected. Furthermore, the recombinant mar ZP3 was antigenic since it was recognized by human ZP3 antibody suggesting that the recombinant mar ZP3 polypeptide expresses major antigens that are shared with the human homologue.

6 Purification and Immunological Characterization of Recombinant Marmoset ZP3

6.1 Introduction

6.1.1 Purification

Rapid advances in recombinant technology have made possible the cloning of almost any gene in a suitable vector to give expression of its product in large quantities. Following a number of planned genetic manipulation steps, the ZP3 proteins, which are difficult to purify in large quantities in their native form, can be synthesized in *E. coli* at a level in excess of 10% of the total cellular protein. Proteins produced by micro-organisms may be intracellular, periplasmic, or secreted into the culture medium. Proteins produced intracellularly have to be released by disruption of the micro-organisms in order to release the protein into the appropriate buffer. The protein can be released by enzymatic, chemical or physical means. Of all the methods employed for this purpose, sonication is probably the most frequently used procedure for the small-scale release of proteins.

Following cell disruption, the first step in the purification of a protein is the removal of cell debris. The separation of solids from liquids is a key operation in enzyme isolation, and is normally accomplished by centrifugation or filtration. The subsequent purification of proteins by chromatography has been a standard laboratory practice for many years. All of the available chromatographic techniques, gel filtration, ion exchange, hydrophobic interaction, affinity, immunoaffinity, and chromatofocusing can be used for the large-scale isolation of proteins.

Separation by ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to an immobilised ion exchange group of opposite charge. The ion exchanger is brought to a starting state, in terms of pH and ionic strength, which allows the binding of the desired solute molecules. When the sample is applied, the molecules carrying the appropriate charge displace counter ions and bind reversibly to the gel. Substances can then be removed from the column by changing the elution conditions, making them unfavourable for the ionic bonding of the solute molecules, by increasing the ionic strength of the elution buffer or changing its pH.

In gel filtration, separation is based on molecular size. The stationary phase consists of porous beads surrounded by a mobile solvent phase. When the sample is applied, the molecules in the mixture partition between the pores in the beads and the solvent. Large molecules are unable to enter the pores and so pass through the interstitial spaces and elute first. Smaller molecules, which can enter the pores, are eluted later, in decreasing order of size.

To date, it has not been feasible to isolate large quantities of native marmoset ZP3 proteins from ovarian material for contraceptive vaccine studies. However, since the amino acid sequences of marmoset ZP3 and human ZP3 are highly conserved (91%), active immunization of marmosets with recombinant mar ZP3 coupled to an immunogenic carrier such as tetanus toxoid should provide valuable information on the way in which an homologous vaccine would behave in the human. In order to conduct such studies large amounts of recombinant marmoset ZP3 would have to be generated. In the studies described in this chapter marmoset ZP3 has been produced by means of a bacterial expression system and the fusion protein has been purified by ion exchange chromatography. The purified material has then been used to generate antibodies in actively immunized rabbits in order obtain preliminary data on the immunogenicity of recombinant marmoset ZP3 *in vivo*.

6. 2 Materials and Methods

6.2.1 Crude Extract Preparation.

The JM105 strain *E.Coli* carrying marZP3 were grown in 20ml rich medium (10g tryptone, 5g Yeast extract, 5 g NaCl, 2 g glucose; after autoclaving sterile ampicillin was added to 100μg/ml) overnight at 37^0C. The following day, 1 litre of rich media was inoculated with 10ml of the overnight cell culture containing the fusion protein. When the cells reached a density of $2x10^8$ cells/ml (A_{600} ~0.5), IPTG was added to a final concentration of 1mM and incubated for 4 hours at 37^0C. The cells were harvested by centrifugation at 4000g for 20 minutes and resuspended in 30ml lysis buffer (20mM Tris-HCl; pH 7.4; 100mM NaCl; 1mM EDTA; 1mM phenyl methyl-sulphonyl fluoride) and stored at 20^0C overnight. The next day, the sample was thawed in cold water, lysozyme (1mg/ml) was added and the mixture incubated on ice for 30 minutes. This material was sonicated using a MSE Soniprep 150 for 3 x 20 seconds, followed by centrifugation at 9000g for 20 minutes. The supernatant (crude extract) was used for further purification.

6.2.2 Anion Exchange Chromatography

DEAE 52, an anion exchanger, (Whatman Laboratory Division, Maidstone, England) was used for initial purification of marmoset ZP3 fusion protein. The anion exchanger was equilibrated with buffer containing 100 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA of pH 7.4. The crude extract (30 ml) was applied to the DEAE 52 column (2.6 x 20 cm column) and eluted with gradient buffer of high ionic strength, containing 300 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA pH 7.4, to lower ionic strength, 100 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA at pH 7.4, at the flow rate of 1ml/minute. The

fractions were analysed on 10% SDS-PAGE and the samples containing marmoset ZP3 were pooled.

6.2.3 Conjugation to Carrier Protein

The anion exchange-purified marmoset ZP3 fusion protein was dialysed against PBS, pH 7.2, overnight. Marmoset ZP3 fusion protein, 2mg in 0.5ml, was mixed with 2mg in 0.5 ml tetanus toxoid. An equal volume of 0.2% glutaraldehyde in PBS was added to the marmoset ZP3 fusion protein and carrier tetanus toxoid and incubated at room temperature for 1 hour. The free amino group was blocked with glycine to a final concentration of 200mM and incubated for 1 hour. The conjugate was dialysed against PBS overnight.

6.2.4 Immunization of Rabbits

Three New Zealand white male rabbits were used for this study. The primary immunization consisted of 50µg marmoset ZP3 fusion protein coupled to tetanus toxoid which was emulsified in an equal volume of Freund's complete adjuvant. An intramuscular injection regimen was used for immunization purposes. Two subsequent booster immunizations were performed at weekly intervals with the same amount of antigen, emulsified with Freund's incomplete adjuvant. One week after the last booster immunization, the rabbits were bled and analysed for their antibody titre.

6.2.5 Electrophoresis and Immunoblotting

Samples of the crude extract from the bacterial cell lysate and anion exchange purified marmoset ZP3 fusion protein were separated by SDS-PAGE and transferred to nitrocellulose membranes as described in sections 5.2.4 and 5.2.5. The nitrocellulose membranes were incubated at room temperature, with 1:200 diluted rabbit sera from the animals immunized against the marmoset ZP3 fusion protein, for 1 hour. The

membrane was washed twice with 0.1M PBS pH 7.2 with 0.1% Tween 20 for 15 minutes. Anti-rabbit IgG conjugated with horseradish peroxidase (Sigma, MO) was employed as a second antibody for 1 hour. Reactivity was visualised by autoradiography using an enhanced chemiluminescence detection system (Amersham International plc, Bucks, England).

6.2.6 Dotblot

Hybond C-Super (Amersham) nitrocellulose membranes were spotted with 1µg of human recombinant ZP3 and marmoset recombinant ZP3 protein and air dried at room temperature. The membranes were soaked in 5% milk powder in Tris-buffered saline, pH 7.6 (TBS) for 1 hour. The following antibody dilutions were then prepared: rabbit anti-recombinant marmoset ZP3 antibody was diluted to 1/500 in a blocking solution, rabbit anti-human ZP3 peptide sequence 341-360 was diluted to 1/2500, rabbit anti-recombinant human ZP3 diluted to 1/500 and rabbit anti-whole human ZP diluted to 1/100. 0.1% Tween-20 was then added and the mixture incubated at room temperature for 2 hours. Washing of the membranes was performed with two brief rinses with distilled water and a 15 minute incubation in TBS with 0.1% Tween-20 (washing buffer) followed by an additional 3 x 5 minutes wash in the same washing buffer. Goat anti-rabbit horse radish peroxide enzyme conjugate diluted to 1/1000 (Sigma) in blocking solution was used as a second antibody with an incubation time of 1 hour at room temperature. The membranes were washed as described earlier and developed with enhanced chemiluminescence (ECL; Amersham) reagents.

6.3 Results

6.3.1 Characterization of Antibodies by Immunoblots

The wet weight of the harvested bacterial cell culture was approximately 5g and was chilled quickly in order to avoid possible degradation. Lysozyme treatment and sonication were employed to break the cells and after centrifugation the soluble solution was removed, which contained the recombinant marmoset ZP3. Using the lysis conditions described in the Materials and Methods section, most, if not all, the fusion protein containing recombinant marmoset ZP3 was present in a soluble form in the lysate (Figure 5.5). The crude extract containing the soluble protein was applied to the DEAE 52 ion exchange column in 100 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA at pH 7.4. The recombinant marmoset ZP3 and other *E. coli* proteins bound to the DEAE-52 column and were then eluted with a linear gradient from a starting buffer comprising 300 mM NaCl, 20 mM Tris-HCl, 1 mM ETDA at pH 7.4. Various fractions were collected and analysed by SDS-PAGE to identify those fractions that contained the marmoset ZP3. Figure 6.1 shows the composition of the different peaks eluted from the DEAE-52 column fractions. Using this procedure the marmoset ZP3 fusion protein could be extensively purified in a single step. The key to this single step purification was to use a narrow range ionic strength gradient elution buffer (Figure 6.2). A variety of alternative chromatographic procedures were employed but this ion exchange strategy proved the most effective. As can be seen from the electrophoresis profiles this procedure generated an extensively purified protein which had not been degraded due to the inclusion of multiple steps in the purification scheme.

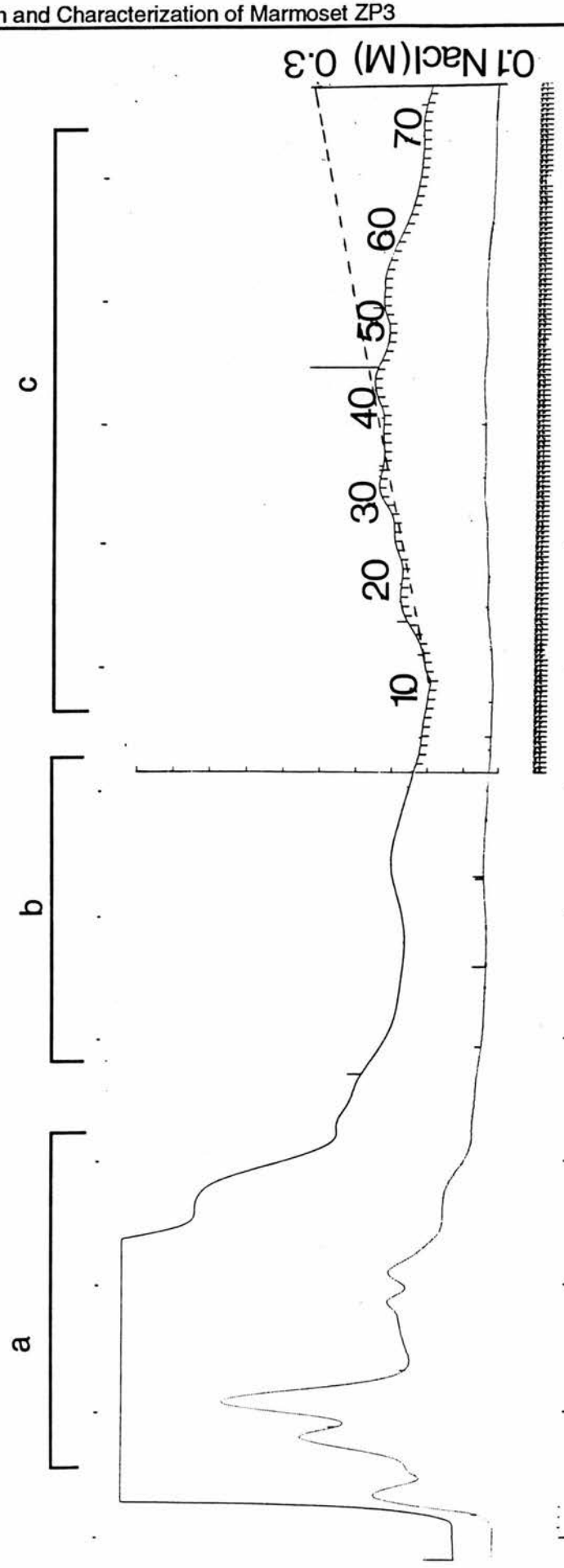


Figure 6.1 A280 profile of eluate from DEAE-52 anion exchange chromatography column during purification of marmoset ZP3 fusion protein from soluble bacterial extract. a, soluble bacterial extract passing through column; b, washing of column; c, elution of marmoset ZP3 fusion protein from the column. The size of the column was 2.6 x 20 cm. and the protein was eluted with gradient buffer of 100-300 mM NaCl, 20 mM Tris-HCl, 1 mM ETDA at pH 7.4. Flow rate of the buffer was 1ml/minute.

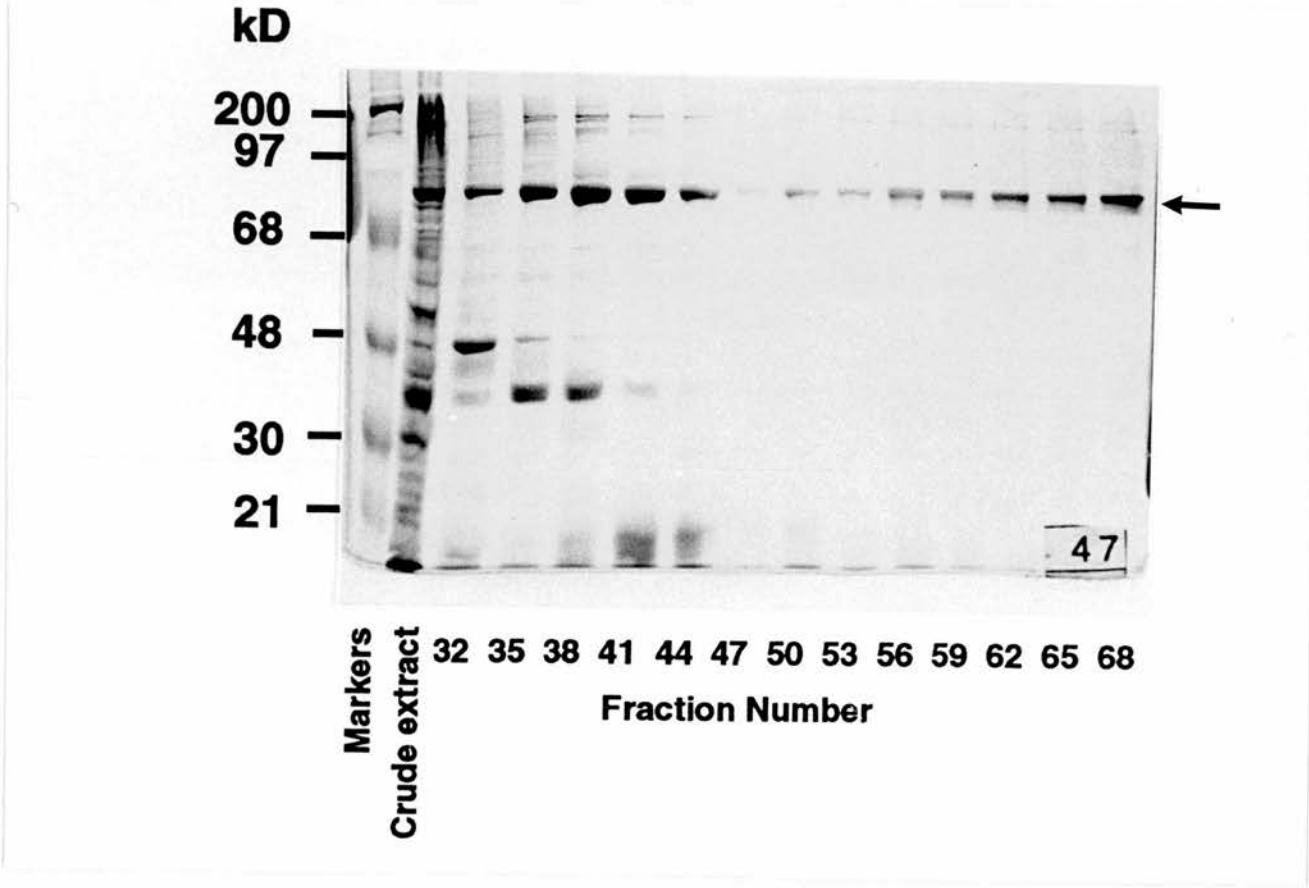


Figure 6.2 Silver stained SDS-PAGE of DEAE-52 fractions of marmoset ZP3 fusion protein. Eluted with gradient buffer of high ionic strength, containing 100-300 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA pH 7.4. Lane indicated as markers on the left hand side contain molecular weight markers, lane indicated as crude extract contain lysed bacterial cell extract that passed through the anion column, lanes 32- 68 contain various fractions that eluted from the anion exchange. Lanes 50-68 contain pure marmoset ZP3 fusion protein, as indicated by an arrow.

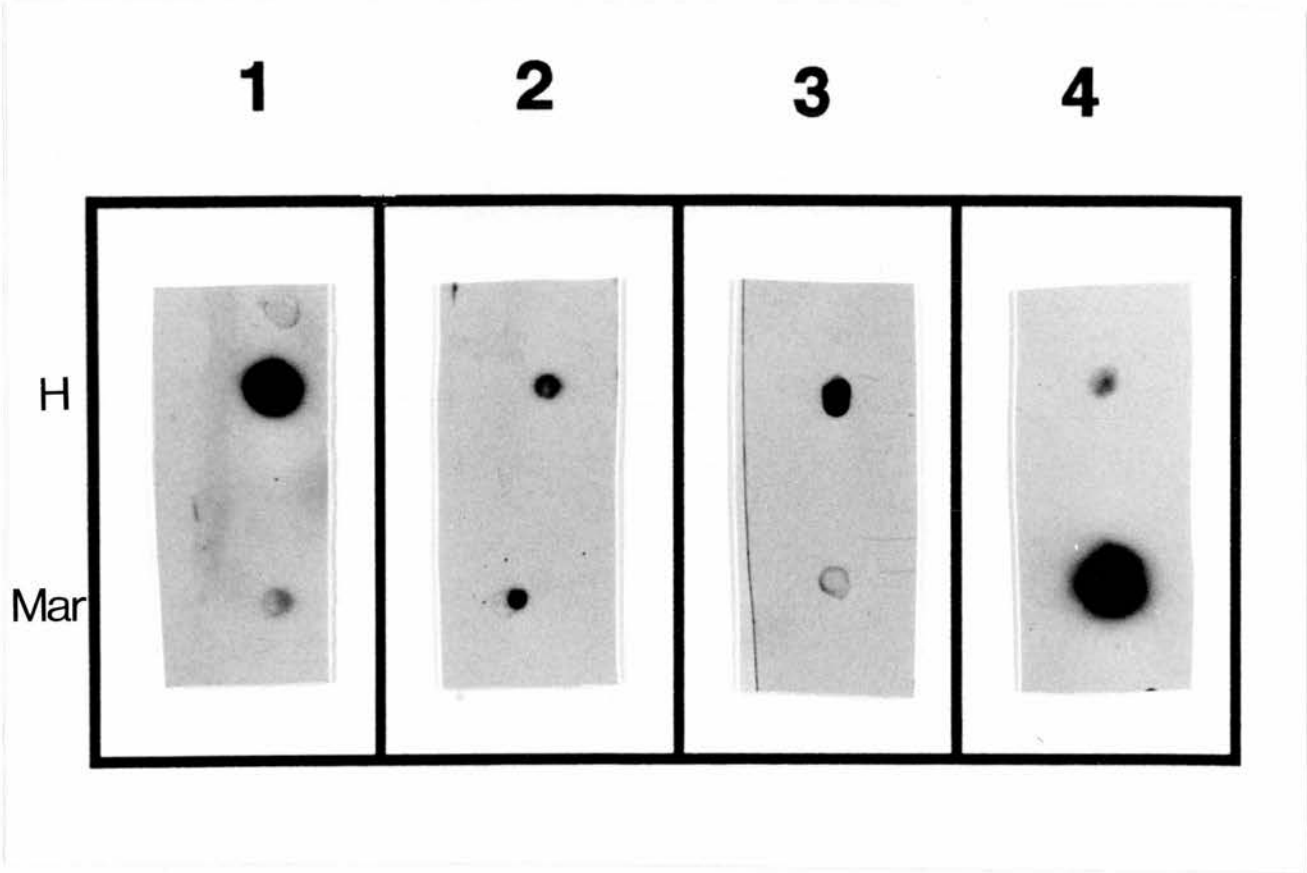


Figure 6.3 Dotblot shows the cross-reactivity of different rabbit antibodies with human (top) and marmoset ZP3 antigens (bottom) dotted onto nitrocellulose membranes. Lane 1 probed with recombinant human ZP3 antibody; lane 2, probed with native human ZP antibody; lane 3 probed with a synthetic peptide antibody against human ZP3 (341-360); lane 4, probed with recombinant marmoset ZP3 antibody.

Preliminary experiments involving the active immunization of rabbits with recombinant marmoset ZP3 failed to produce significant antibody titre. In order to enhance the immunogenicity of the recombinant marmoset ZP3, conjugation with tetanus toxoid was carried out. Single step glutaraldehyde coupling was employed to conjugate the carrier molecule. After the primary immunization and two booster injections with conjugated recombinant protein, antibody levels could be visualised with the immunoblot procedure. The antibody developed against recombinant marmoset ZP3 fusion protein was found to recognize this molecule in both the crude extract and the partially purified fusion protein in immunoblot experiments.

Rabbit antibodies to recombinant marmoset ZP3 proteins were further characterised by immunoblotting using recombinant human and marmoset ZP3 proteins generated in CHO cells as the target. Figure 6.3 shows the cross-reactivity of the rabbit anti-marmoset ZP3 fusion protein with human and marmoset ZP3 antigens dotted onto nitrocellulose membranes. It was observed that the recombinant marmoset ZP3 antibody specifically recognized both recombinant human and marmoset ZP3 generated in an alternative, CHO expression system. This antibody recognized marmoset ZP3 more strongly than the human ZP3, as shown in Figure 6.3. An anti-human recombinant ZP3 antibody also recognized recombinant marmoset ZP3, but the signal was not as strong as that produced with the homologous human protein. In contrast, the antibody produced against whole native human ZP could detect both human ZP3 and marmoset ZP3 with equal intensity. An antibody directed against the human ZP3 amino acid sequence 341-360 could detect recombinant human ZP3 and recombinant marmoset ZP3, but exhibited greater cross reactivity with the former.

6.4 Discussion

The ZP plays a critical role in sperm-egg interaction during fertilization. As such, it is a potential target site for the development of an effective immunocontraceptive vaccine. It is well recognized that mammalian ZP glycoproteins are complex antigens and that their immunogenicity and antigenicity vary between species (Dunbar *et al.*1989; Millar *et al.*1989). Immunization with ZP3 antigens can result in an autoimmune oophoritis leading to the destruction of primordial and growing follicles as well as altered reproductive cycles (Rhim *et al.*1992; Skinner *et al.*1984; Paterson *et al.*1992). However, immunization with a mouse ZP3 synthetic peptide containing only a B cell epitope produced infertility without ovarian pathogenicity (Millar *et al.*1989). In light of these results, it is important to define specific antigenic domains which may induce antibodies that interfere with different stages of fertility, e.g., inhibition of sperm binding and penetration, or the inhibition of follicular development, or the disruption of oocyte maturation. In order to determine whether the marmoset monkey would be a suitable model to study such events it is necessary to determine how this species responds to the induction of immunity against the homologous zona pellucida antigen. In order to facilitate such a study an investigation has been conducted into the generation of bacterially expressed recombinant marmoset ZP3 proteins which have then been purified and used to generate anti-ZP3 antibodies

In order to achieve this objective a full-length marZP3 cDNA was subcloned in the pMAL™-c vector (New England Biolab, Herts, England), downstream of the *malE* gene which encodes maltose binding protein (MBP) and results in the expression of an MBP fusion protein. The strong "tac" promoter and the *malE* translation initiation signal present upstream of the cloned marZP3 gave high-level expression of marZP3. Antibodies directed against native human ZP, recombinant human marmoset ZP3

expressed in CHO cells and a human ZP3 peptide containing the amino acid sequence 341-360 could all recognize the recombinant marmoset ZP3 polypeptide to varying degrees.

Preliminary immunization experiments with the purified recombinant marmoset ZP3 in rabbits indicated that this material was not immunogenic. This might, in part be expected, because it has been established that deglycosylated ZP proteins are less immunogenic than the native or partially glycosylated proteins (Bhatnagar *et al.*1992; Keenan *et al.*1991; Millar *et al.*1989). It is also likely that the molecular nature of the recombinant ZP3 affects its antigenicity. Thus, a mouse recombinant ZP3 fusion protein containing ß-galactosidase generated a weak immune response in female mice and did not react with the ZP in frozen sections of mouse ovaries (Dean *et al.*1989).

Rabbits immunized with the purified recombinant marmoset ZP3 fusion protein conjugated to tetanus toxoid produced antibodies that recognized human recombinant ZP3 on ELISA and dotblot assays. This enhanced immunogenicity of the recombinant marmoset ZP3 after conjugation to tetanus toxoid was due the helper T cell epitopes provided by the carrier molecule. This demonstrates the possibility of using such recombinant ZP:carrier protein conjugates for active immunization studies rather than the native ZP proteins and glycoproteins that have been used in the past (Henderson *et al.*1987a; Henderson *et al.*1987b; Paterson *et al.*1992). The recombinant marmoset ZP3 antibody recognized the recombinant marmoset ZP3 and recombinant human ZP3 on dotblot assays. These observations are of interest, and expected, since the amino acid sequence of marmoset ZP3 and human ZP3 exhibits 91% homology. It was also interesting to note that the recombinant marmoset ZP3 antibody recognized recombinant marmoset ZP3 more strongly than the recombinant human ZP3. An explanation for this weak reactivity could be that the antibody was

directed towards a dominant epitope of marmoset ZP3 which might be absent in human ZP3. The fact that recombinant human ZP3 antibody cross-reacted with the recombinant human ZP3 more strongly than the recombinant marmoset ZP3 is also a reflection of antigenic differences between the human and marmoset ZP3 proteins despite their sequence homology. The anti recombinant hZP3 antibody could have recognized a different epitope of marmoset ZP3 or the same epitope might have been configured in a slightly different way. The fact that antibody generated against native human ZP recognized recombinant marmoset and human ZP3 equally well suggests that in these recombinant proteins the conformation of the ZP3 epitopes was different from the native protein. Different antibodies to ZP have a variety of specificities since they may target peptide, conformational, and carbohydrate portions of the ZP structure (Drell & Dunbar, 1984; Maresh & Dunbar, 1987; Keenan *et al.*1991). Thus there are number of different levels of epitope recognition at which differences in the antigenicity of the recombinant ZP proteins might be expressed.

The use of a recombinant ZP3 immunogen offers several experimental advantages over previous approaches to the induction of immunity against the zona pellucida. ZP glycoproteins manifest great heterogeneity on electrophoretic gels, so that it is not always possible to identify or characterize different ZP proteins or antigens by electrophoretic methods. Even enzymatic or chemical deglycosylation procedures do not eliminate all carbohydrate structures so that some heterogeneity remains even after such treatments. As a consequence of the presence of charged isomers, it has been difficult to distinguish the core ZP proteins or to be certain that these proteins have been purified to absolute homogeneity. The use of recombinant ZP3 avoids any possible complications due to low levels of contaminating proteins from the oocytes and granulosa cells, or from other ZP proteins. For this reason, recombinant ZP proteins are clearly superior antigens to those raised against native

material in terms of their purity and as such should permit more precise analysis of the immunogenicity and antigenicity of these molecules. Against this has to be balanced the fact that the recombinant material may not faithfully replicate the antigenic structures expressed by the native protein.

6.5 Summary

The recombinant mar ZP3 produced in soluble form could be easily purified to homogeneity using anion exchange column. Moreover, a construct including recombinant marmoset ZP3 conjugated to tetanus toxoid was found to be immunogenic. The antibodies generated using this construct recognized recombinant marmoset and human ZP3 in dotblot assays. The use of such recombinant ZP3 in active immunization studies would eliminate any possible complications due to low level of contaminating proteins from the oocytes and granulosa cells. Furthermore, the abundant availability of pure recombinant mar ZP3 will facilitate the design of large scale studies for accessing the safety and efficacy of contraceptive vaccines that target the zona pellucida.

7 Active Immunization of Marmoset with Porcine ZP3 Antigen

7.1 Introduction

Previous studies have demonstrated that active immunization with ZP preparations can induce infertility in a variety of different animal models including bitches (Mahi Brown *et al.*1988), rabbits (Skinner *et al.*1984), squirrel monkeys (Sacco *et al.*1991) and marmoset monkeys (Paterson *et al.*1992). Every species examined exhibited abnormal reproductive cycles characterized by a fall in circulating progesterone levels (Paterson *et al.*1992; Skinner *et al.*1984; Mahi Brown *et al.*1988). It was also found that those animals developing the highest titres of anti-zona antibodies following the induction of immunity with porcine ZP developed abnormal oestrous cycles characterized by elevated oestrogen and low progestrone values suggesting a failure of ovulation (Mahi Brown *et al.*1985; Skinner *et al.*1984; Sacco *et al.*1991). It was postulated that the most likely reason for this side effect lay in the impurity of zona preparations (Gulyas *et al.*1983a), and that purified ZP preparations or ZP components specific for sperm receptor activity (ZP3) could help to overcome this problem (Gulyas *et al.*1983a; Sacco *et al.*1986a).

In order to undertake such studies, the largest zona pellucida glycoprotein (ZP1) was purified by preparative isoelectric focusing (Dunbar *et al.*1989; Paterson *et al.*1992) while ZP3 was purified by a series of chromatography steps (Sacco *et al.*1983; Sacco *et al.*1987; Paterson *et al.*1992). Whatever the nature of the zona components used for the induction of immunity, and irrespective of the purification procedures employed, active immunization with these purified ZP glycoproteins was

still associated with the eventual appearance of ovarian dysfunction. The reasons for this pathological response to the induction of immunity against the zona pellucida remain unclear but the issue is the subject of intense speculation since it is of major relevance to the development of a clinically useful contraceptive vaccine.

Recent analyses of this problem suggested that the carbohydrate content of the ZP3 glycoprotein is the main inducer of ovarian pathogenicity (Keenan *et al.*1991; Jones *et al.*1992) Rabbits immunized with the partially deglycosylated porcine ZP3 constituent, ZP3å, as well as the chemically deglycosylated peptide backbones of porcine ZP3å and ZP3ß, showed no significant elevations of their circulating gonadotrophin concentrations and continued to display a normal progesterone response to exogenous hCG administration (Keenan *et al.*1991). Moreover, the ovarian histology in these animals showed no signs of the pathological changes associated with the induction of immunity against fully glycosylated ZP3 (Jones *et al.*1992). In contrast to these findings in the rabbit, marmosets immunized with the chemically deglycosylated peptide backbone of porcine ZP3ß did exhibit ovarian dysfunction, although this pathology took two years to manifest itself. Since this pathology was associated with the depletion of the primordial follicle pool, this side effect did not reverse as antibody titres subsequently declined (Paterson *et al.*1992).

Using the mouse as an experimental model, important evidence has been obtained to indicate that the disruption of ovarian function following the induction of immunity against the zona pellucida might be due to T-cell epitopes within the ZP3 sequence. Absence of T-cell epitopes from a ZP3 peptide resulted in an immunogen that could induce infertility without any pathological effect on the ovary (Millar *et al.*1989). Conversely, ZP3 peptides as small as 8 amino acids in length could be used to induce ovarian pathology (oophoritis) in the absence of anti-zona antibodies providing T-cell epitopes were present. Since adoptive transfer of $CD4^-$ T-cell lines

derived from actively immunized animals caused oophoritis in naive recipients, in the absence of an antibody response against the zona pellucida, T-cell mediated immunity is clearly the mediator of ovarian pathology in this particular model (Rhim *et al.* 1992). It should be noted however that these studies were conducted in a strain of mouse ($B6AF_1$) that is particularly susceptible to T-cell mediated autoimmune disease. The extent to which this model applies to the ovarian pathology observed in other species following the induction of immunity against the zona pellucida is unknown.

In view of the importance of this pathology to contraceptive vaccine development a detailed analysis has been undertaken of this process in the marmoset monkey. In this chapter I shall describe an analysis of both the nature and kinetics of the pathological responses observed in marmoset monkeys actively immunized with glycosylated porcine ZP3. This information was then used to orientate subsequent passive immunization studies in which the importance of the humoral response to ZP3 antigens in the aetiology of ovarian dysfunction was demonstrated.

7.2 Material and Methods

7.2.1 Purification of Porcine ZP3

Porcine ovaries were collected from the slaughter house and stored at -20°C until further processing. Porcine oocytes were obtained by mincing thawed ovaries in a meat grinder with copious amounts of ice-cold saline (0.9%) followed by sieving of the homogenate through nylon screens of decreasing size (500 to 75µm). The isolated oocytes were further purified by centrifugation in a discontinuous Percoll gradient (40%, 20%, 10% bottom-top) for 30 minutes (2000 x g, 20°C). The oocytes collected from the 10-20% interface were washed free of Percoll with PBS (pH 7.4) and then gently disrupted in a small glass homogenizer, after which the remnants of the oocytes

were removed in the supernatent following centrifugation. The purified ZP were then heat solubilized at 78°C for 20 min and finally ultracentrifuged (100 000 x g, 90 min, 4°C). The resulting supernatant was dialysed against several changes of 0.1 M ammonium hydrogen carbonate (5L). The heat solublized ZP were treated with 2% SDS and applied to a Sephacryl S-400 column equlibrated with 25 mM sodium phosphate buffer (pH 7.2; 0.1% SDS, 0.02% sodium azide) to obtain a ZP3-enriched fraction. This fraction was further purified by the use of hydroxylapatite chromatography employing a linear gradient of 10-500 mM sodium phosphate, pH 6.8, containing 0.1% SDS. Renaturation of porcine ZP3 was carried out in the presence of 6 M guanine hydrochloride.

7.2 2 Active Immunization

Marmoset monkeys from the Primate Centre of the Medical Research Council were used for this study. An immunization schedule was designed that would generate a sudden surge of antibody at a defined time point so that a time-dependent analysis of the changes in ovarian histology could be undertaken Three marmoset monkeys were sensitized with 100 µg of purified porcine ZP3 in peanut oil and muramyl dipeptide adjuvant, adsorbed with alum and allowed to rest for 8 months. These animals then received a booster immunization containing the same amount of antigen in non-ulcerative Freund's adjuvant adsorbed on alum in order to produce a sudden synchronized surge of anti-zona antibodies. A second group of 3 control animals received vehicle alone.

7.2.3 ELISA Assay

Blood samples were taken weekly to monitor antibody titres using an enzyme linked immunosorbent assay (ELISA). The ELISA plates were coated with 50 µl of a

solution containing 10 µg/ml heat-solubilized porcine ZP in 0.1 mM bicarbonate buffer pH 9.0 and allowed to adsorb at 4°C for 16 hours. Plasma samples (50 µl) were added in doubling dilution from 1/10 to 1/20,480 and incubated at room temperature for 2 hours. The plates were washed three times with PBS containing 0.05% Tween-20; then 50µl of a 1/1000 dilution of goat anti-human IgG alkaline phosphatase conjugate was added to each well and incubated for 2 hours at room temperature. The plates were washed with PBS containing 0.05% Tween-20; then 50 µl of substrate solution was added (1 mg/ml p-nitrophenylphosphate in 10% diethanolamine, pH 9.8) and allowed to develop in the dark for 1 hour at room temperature. The reaction was terminated with 50 µl 3 M NaOH, and the absorbency at 405 nm was measured on a Microtek ELISA plate reader. The end point for the assay was an absorbency twice that of the negative control reading. The antibody titres for each animal were monitored on weekly blood samples.

7.2.4 Histology

A laparotomy was performed on each of the 3 treated marmoset monkeys and the right ovary was removed 2, 4, and 8 weeks after the booster injection; the left ovary was subsequently removed and autopsy performed 16, 24 and 32 weeks post injection. All the ovaries were fixed in 4% paraformaldehyde in PBS pH 7.4, embedded in paraffin and serially sectioned to generate 5µm tissue slices. Every 20th section was stained with haematoxylin and eosin and the primordial follicles were counted by light microscopy. Primordial follicles were defined as those structures possessing oocytes surrounded by one layer of squamous prefollicular cells.

7.3 Results

7.3.1 Antibody Titre

A peak in ELISA antibody titres (1:1280 to 1:10240) was attained following the initial immunization and occurred approximately 17-20 days after the first injection (Figure 7.1). This peak was followed by a gradual decline in titre to reach basal levels at around 220 days. All three animals had comparable antibody titres after the primary immunization. On day 245 the first booster immunization was given and the antibody titre rapidly reached a maximum titre of 1:3840 and this peak was once again followed by a small decline, although the titres were subsequently maintained at 1: 640 until the end of the study.

7.3.2 Histology

The first ovary recovered from an actively immunized animal two weeks after the administration of a booster immunization exhibited completely normal ovarian histology. The primordial follicle count was exceptionally high (1:9614) in this animal and a large number of actively growing primary, secondary and Graafian follicles were present (Figure 7.3). The ovarian sections examined from this animal also revealed the presence of a corpus luteum. Significantly, there was no evidence of infiltration of mononuclear cells in the ovary.

The ovarian material obtained from the marmosets at later time points revealed a dramatic loss of primordial follicles as early as 4 weeks after the administration of a booster injection. Only 265 primordial follicles were present in the ovary removed at this stage although actively growing primary and secondary follicles were also observed, as well as an apparently normal corpus luteum (Figure 7.4 and 7.5).

This disruption of primordial follicles was progressive and was marked from 4 weeks following the booster injection onwards. The loss of primordial follicles was also severe in that there were very few primordial follicles present in the ovarian cortex in the animals examined from the fourth week. There was also little time dependency in the loss of primordial follicles, since the numbers decreased dramatically within the first 4 weeks following the administration of a booster injection and remained low thereafter. Histological evaluation failed to reveal any evidence of leucocyte infiltration over this period. The ovary removed 16 weeks after the booster injection showed (Figure 7.6) very few primordial follicles (59) compared with the ovary removed from the same animal at two weeks (9614). Despite the dramatic loss of primordial follicles the ovarian sections showed evidence of primary and secondary follicles.

The autopsies performed 16, 24, and 32 weeks after the booster immunization of the animals revealed very low numbers of primordial follicles: 59, 123, and 23, respectively. Despite the dramatic loss of primordial follicles there were, even at these late stages, primary and secondary follicles in the ovaries as well as luteal tissue (Figure 7.7).

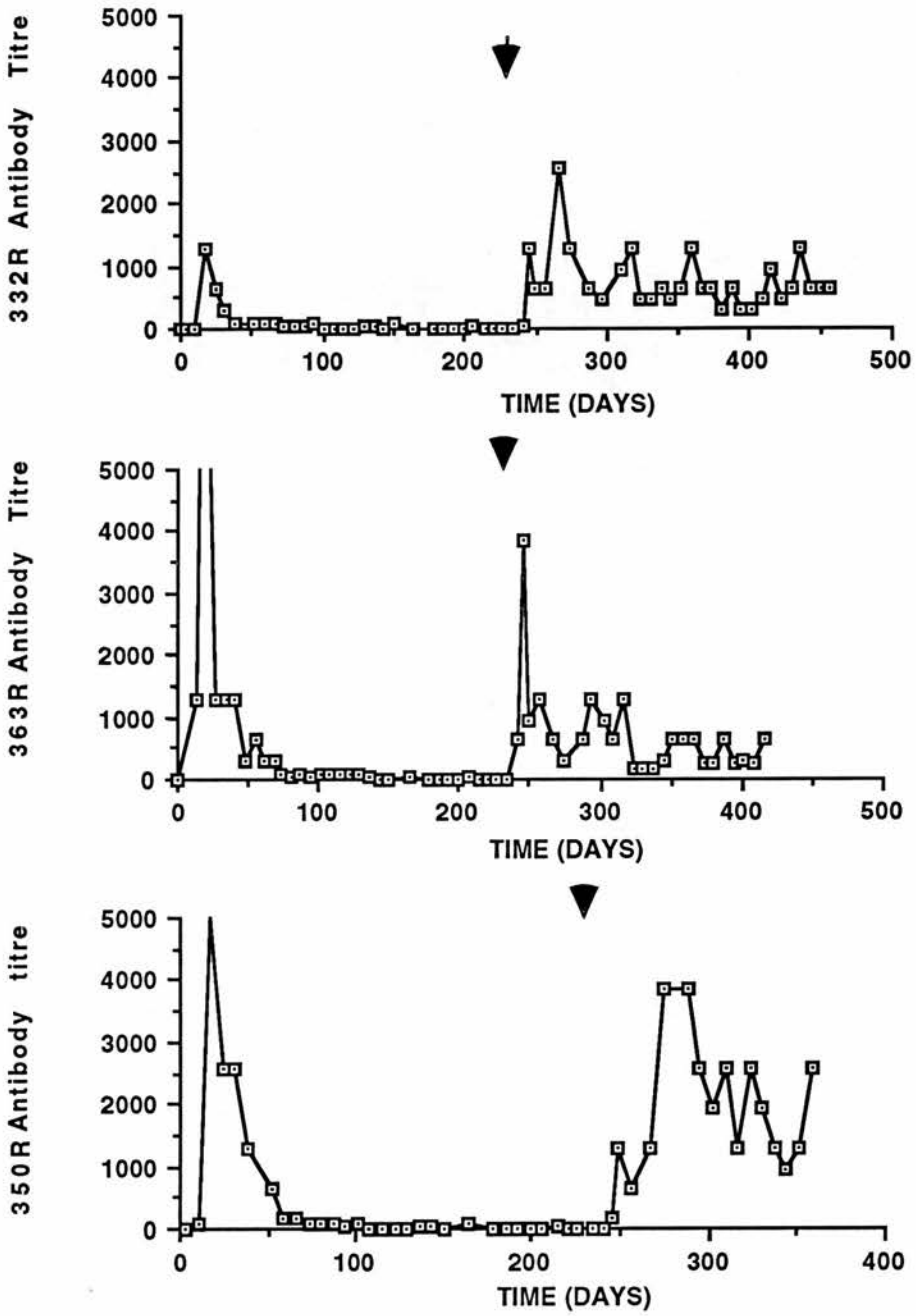


Figure 7.1 Antibody titre of actively immunized animal analysed by ELISA. Arrows indicate the time of booster immunization.

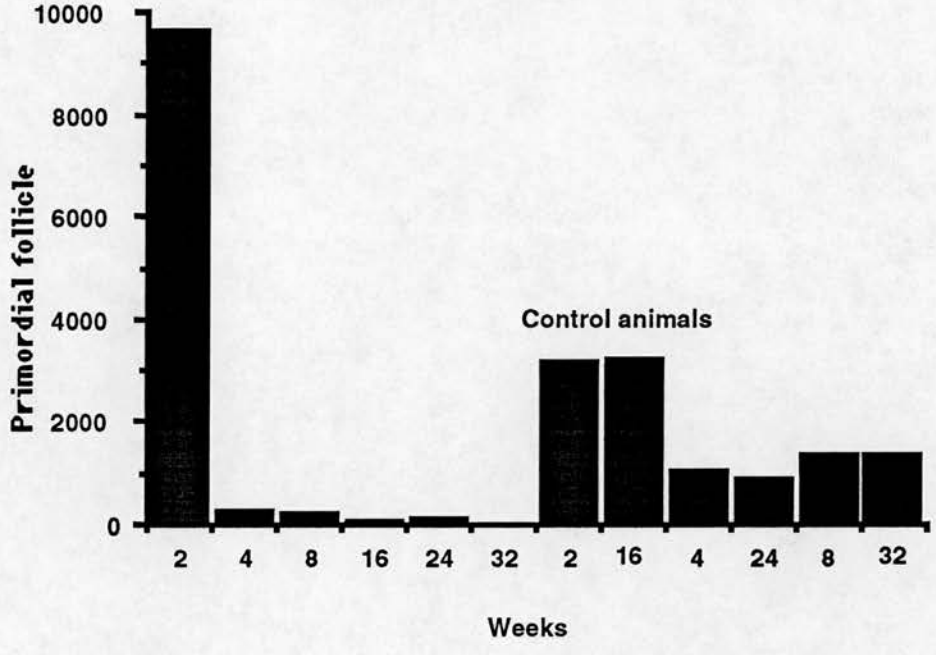


Figure 7.2 Effect of active immunization on the primordial follicle population of marmoset ovaries at different times. In the control, each animal's left and right ovary primordial follicles counts are presented together even though the ovaries were removed at different time to indicate there are no discrepancy in primordial follicle counts.

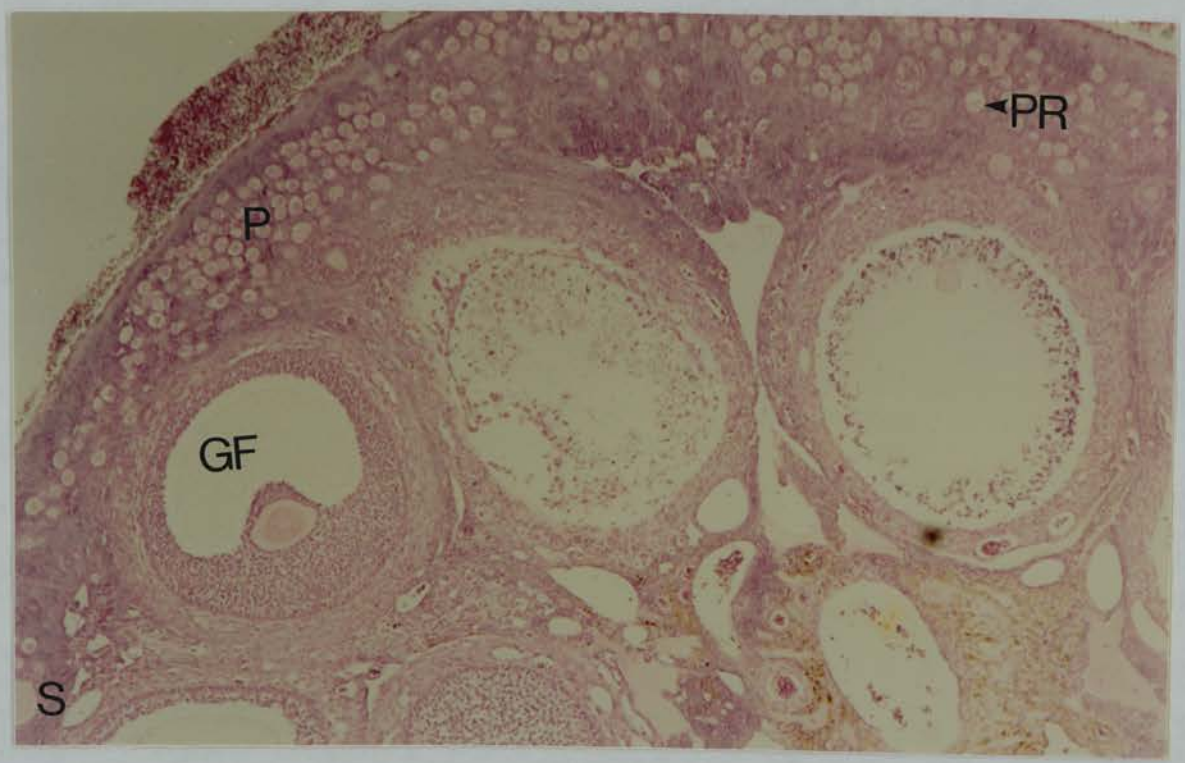


Figure 7.3 Histology section of marmoset ovary immunized with porcine ZP3 antigen. Marmoset ovarian section stained with haematoxylin and eosin. The number of primordial follicles (P) are very high even after 2 weeks of postbooster immunization. Many primary (PR), secondary (S) and Graafian follicles (GF) are present. (10 x)

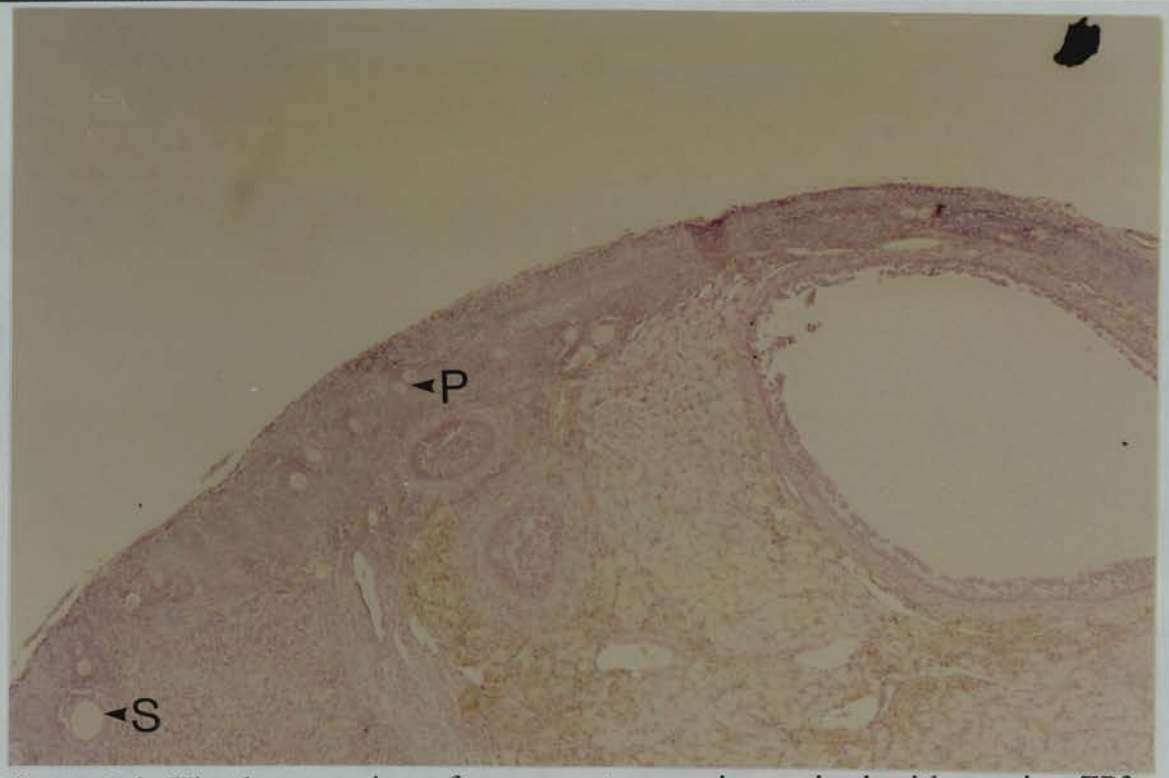


Figure 7.4 Histology section of marmoset ovary immunized with porcine ZP3 antigen. Marmoset ovarian section stained with haematoxylin and eosin. After four weeks of postbooser immunization there are few primordial follicles (P) and secondary follicles (S). (10 x)

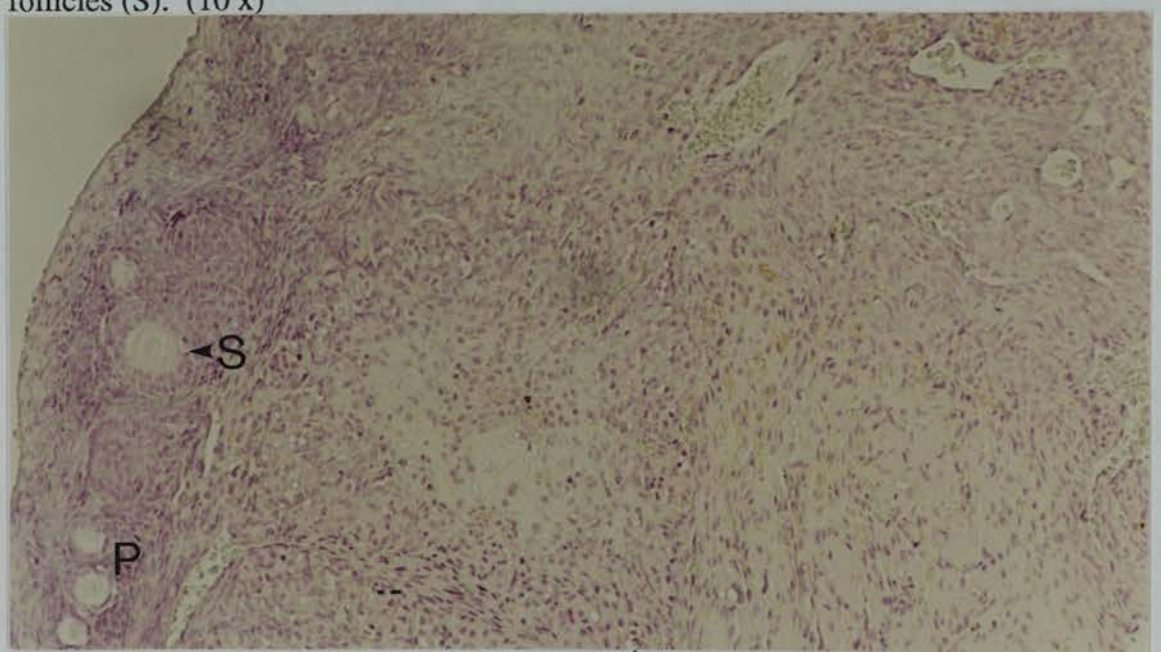


Figure 7.5 Histology section of marmoset ovary immunized with porcine ZP3 antigen. Marmoset ovarian section stained with haematoxylin and eosin. After four weeks of postbooser immunization there are few primordial follicles (P) and secondary follicles (S). (25 x)

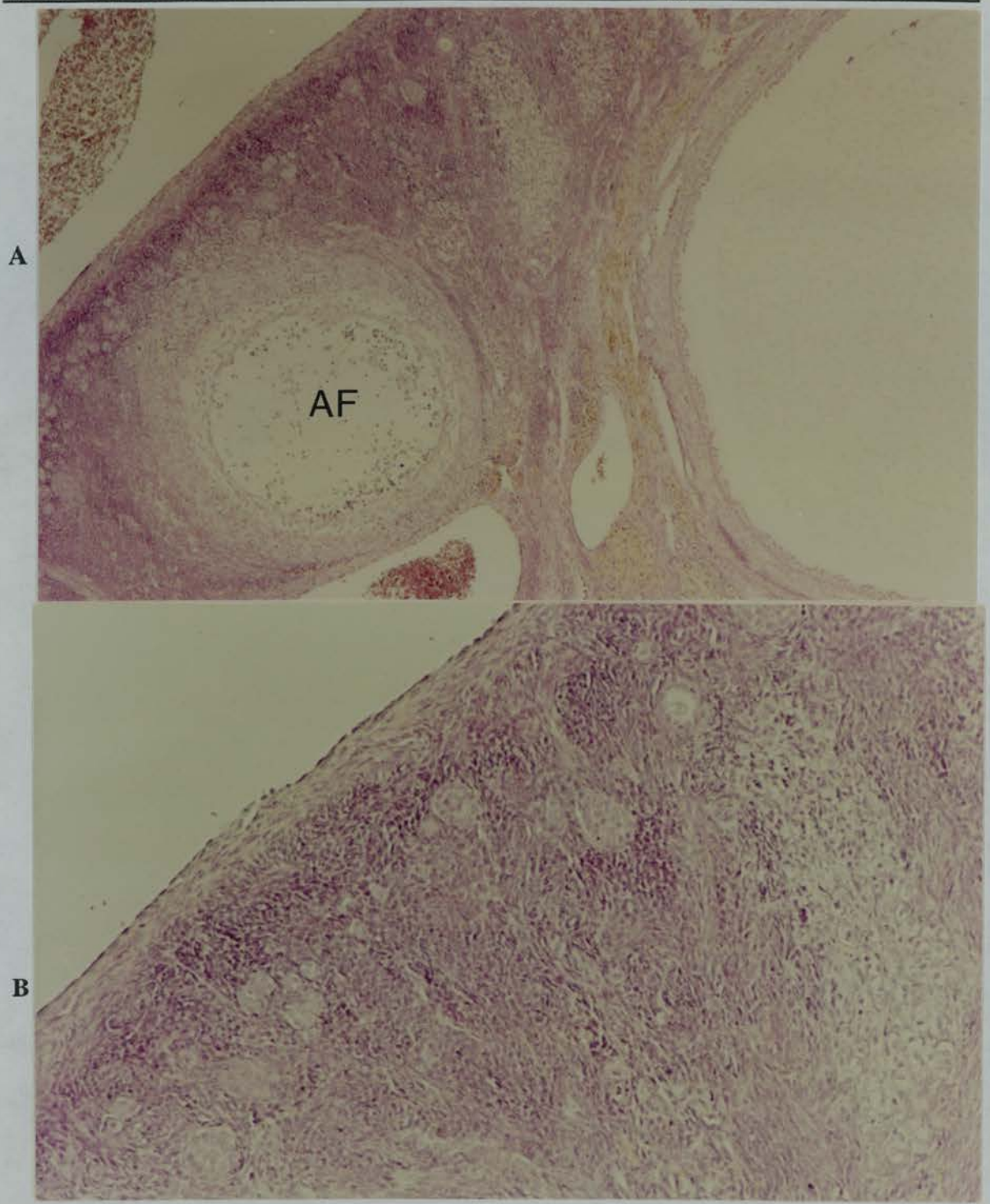


Figure 7.6. Histology section of marmoset ovary immunized with porcine ZP3 antigen. Marmoset ovarian sections stained with haematoxylin and eosin. The ovary removed 16 weeks after the postbooster injection showed very few primordial follicles and there is evidence of atretic follicle (AF). (B, 25 x) High magnification of (A, 10 x).

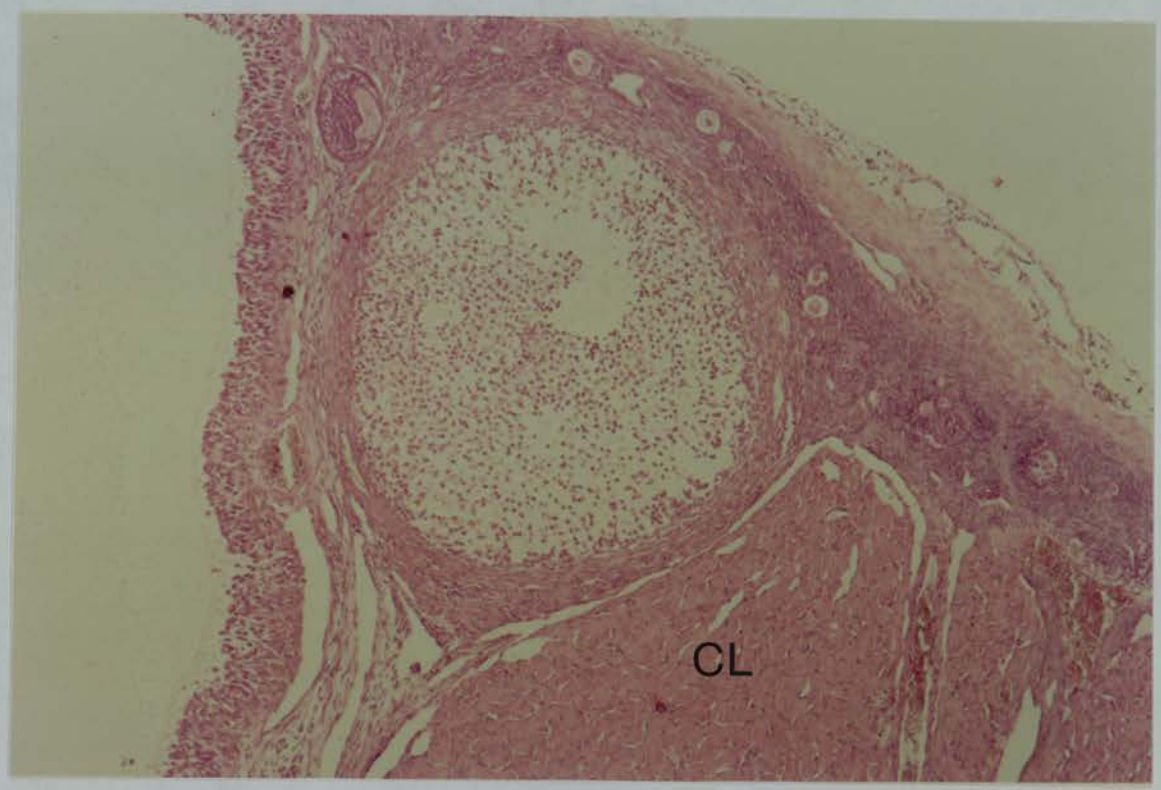


Figure 7.7 Histology section of marmoset ovary immunized with porcine ZP3 antigen. Marmoset ovarian sections stained with haematoxylin and eosin. The ovary removed 24 weeks after the postbooster injection showed very few primordial follicles and there is evidence of luteal tissue (CL). (10 x)

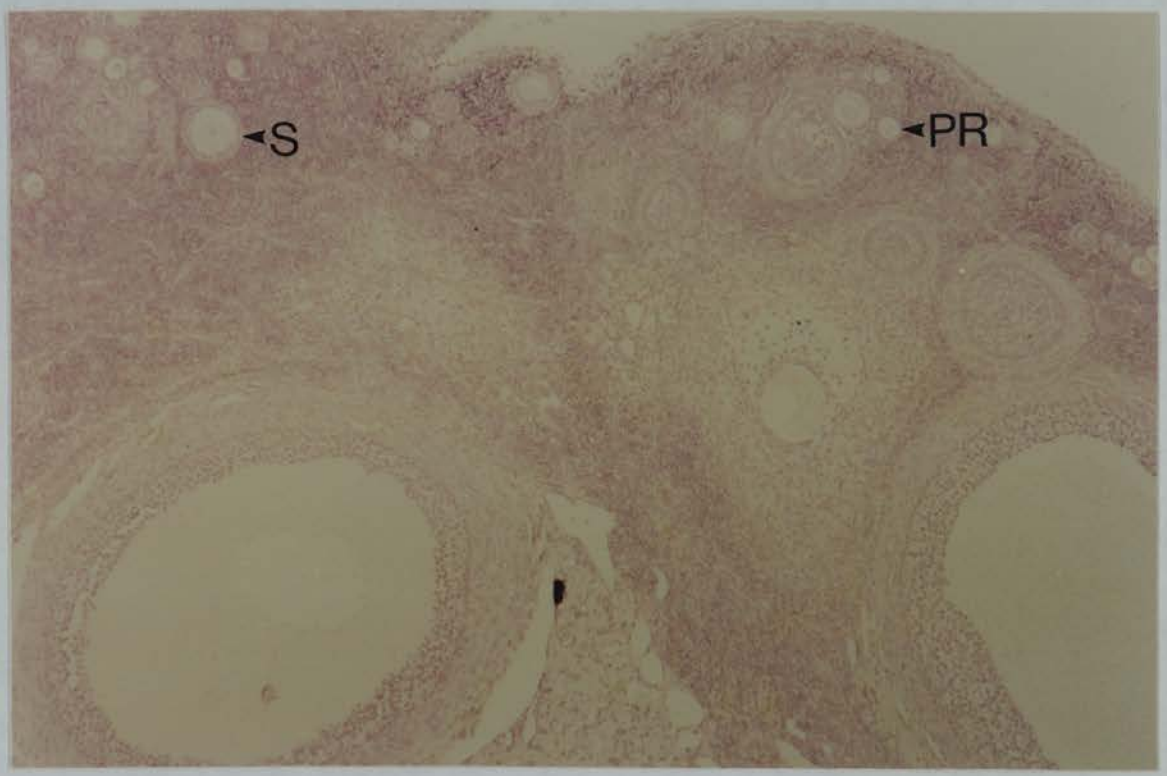


Figure 7.8. Marmoset ovarian section stained with haematoxylin and eosin. The ovaries of the control animals appeared normal with numbers of primordial, primary (PR) and secondary follicles (S). (10 x)

The ovaries of the control animals appeared normal with large numbers of primary and secondary and growing follicles, and corpora lutea. (Figure 7.8) The numbers of primordial follicles recorded in the individual ovaries were consistent for each animal and were not influenced by the sham immunizations carried out with vehicle alone (Figure 7.2). Thus in the control animal, number 312R, the primordial follicle count for the right ovary was 3160 at 2 weeks and 3228 for the left ovary at 16 weeks. Similarly the control animal, number 392R, exhibited a right ovary primordial follicle count of 1060 at 4 weeks compared with 888 for the left ovary at 24 weeks. The control animal number, 399R, possessed 1284 primordial follicles in the right ovary at 8 weeks compared with 1368 in the left ovary at 32 weeks.

7.4 Discussion

The potential of the ZP as an antigen for contraceptive vaccine development has been clearly demonstrated in various animal model studies. In order to be acceptable any contraceptive vaccine would have to be free of untoward side effects. At the present time, there are clear problems with the use of the ZP as a target antigen in contraceptive vaccine development due to the ovarian pathology that can occur following the induction of immunity against this structure. Existing data indicate that active immunization against the native ZP3 glycoprotein is associated with the appearance of an ovarian pathology characterized by the accelerated disappearance of primordial follicles from the ovarian cortex (Skinner *et al.*1984; Keenan *et al.*1991). The development of an immune response against ZP not only affects the ovum but also the ovarian endocrine system; and thence indirectly the hypothalamo-hypophyseal-ovarian axis leading to changes in the circulating levels of gonadotrophins and steroids (Skinner *et al.*1984; Sacco *et al.*1987; Mahi Brown *et*

*al.*1985). In these studies (Skinner *et al.*1984; Keenan *et al.*1991) a marked decrease in primordial and developing follicles was mirrored by elevations in LH and FSH and by a loss of hCG-induced progesterone secretion. Such disruptions of ovarian physiology could theoretically result from humoral or cell-mediated immune responses to ZP antigens (Shivers *et al.*1981; Mahi Brown *et al.*1992; Rhim *et al.*1992).

The experiments discussed in this chapter reveal that the active immunization of female marmoset monkeys with porcine ZP3 results in a profound alteration of ovarian follicular development and a rapid depletion of the primordial follicle pool. This reduction in the number of primordial follicles was observed as early as 4 weeks after the induction of high titre antibodies against the zona pellucida. Despite this reduction in the size of the primordial follicle population there were actively growing primary and secondary follicles in these ovaries as well as corpora lutea. The simultaneous decrease in primordial follicle counts and the presence of other growing follicles suggest that the former must be highly susceptible to immunological damage. Similar observations have been made in different animal models such as the rabbit (Skinner *et al.*1984), marmoset (Paterson *et al.*1992), and baboon (Dunbar *et al.*1989).

The aetiology of primordial follicle population depletion following the induction of immunity against the ZP is not clear. It might be reasonable to suppose that antibodies against ZP3 would induce an interruption of follicular development coincident with the stage at which the ZP first appears as an extracellular matrix between the oocyte and follicular cells. Clearly the primordial follicle does not secrete zona pellucida antigens in a major way since, by definition, the oocyte in a primordial follicle is not surrounded by a zona pellucida. However, whether the oocytes in such follicles exhibit a low level of ZP antigen expression is an extremely controversial issue. Certainly in the rabbit there is now good evidence that the 55 kD ZP antigen is secreted by both the follicular cells and the oocyte and that low level expression is

observed in the primordial follicles (Lee & Dunbar, 1993). However, studies of ZP3 mRNA transcription and translation in the mouse and marmoset (this study) clearly indicate that these activities are not observable until an oocyte has been recruited from the primordial follicle population and has entered the actively growing follicular pool.

By 8 weeks post-immunization, the number of primordial follicles was severely reduced (187). After 16, 24, and 32 weeks of immunization the number of primordial follicles was 59, 123 and 23 respectively. This shows that after the intial decline in primordial follicle number observed at eight weeks, there was no further reduction in the size of this follicle population over the ensuing 24 weeks.

Dunbar *et al* (1989) found histological evidence of primordial follicle depletion 196 days after the induction of immunity against the Mr=80 000 zona polypeptide in baboons. Furthermore, in this study the complete depletion of the primordial follicle population was noticed 32 weeks (224 days) postimmunization. The condition of the ovary was thought to resemble premature ovarian failure in women, a phenomenon which may have an autoimmune aetiology. Thus, it has been suggested that this form of infertility in women may be due to the presence of antibodies that react with granulosa cells secreting zona antigens and thereby damage this cell type (Wolgemuth *et al.*1984). However the evidence obtained in this study suggests that the granulosa cells do not express ZP3 antigens and retain sufficient structural integrity.

The other indirect post-immunization ovarian dysfunction which has been recorded is depressed progesterone secretion in several species, including rabbits, (Skinner *et al.*1984; Keenan *et al.*1991) dogs (Mahi Brown *et al.*1988; Mahi Brown *et al.*1985), baboon (Dunbar *et al.*1989), and marmoset (Paterson *et al.*1992). Rabbits immunized with SIZP, ZP3, and ZP3ß/EBGD showed a significant elevation of LH and FSH and a significant decline of peak progesterone levels. In contrast, rabbits immunized with ZP3å/EBGD, ZP3å/DG, and ZP3ß/DG showed no significant

elevations of gonadotrophins and continued to display cyclic progesterone secretion in response to hCG (Keenan *et al.*1991). Porcine ZP3 immunization in squirrel monkeys resulted in initial steroid hormonal disturbances, but subsequently ovarian function returned to normal in all the animals examined (Sacco *et al.*1987). It has been also suggested that the ovarian pathogenesis was due to the immunogenicity inherent in the carbohydrate moiety of the ZP preparations and use of deglycosylated zona molecules produced normal cyclic progesterone secretion. On the contrary, Paterson *et al* (1992) used chemically deglycosylated porcine ZP3ß in marmoset monkeys and observed the appearance of ovarian pathology as evidenced by a depletion of the primordial follicle population, appearance of follicular clusters and no evidence of growing follicles. In the same study all the marmoset monkeys displayed cyclic progesterone secretion for a period of 200 days which was associated with the persistence of a few primordial follicles up to that time, which were still competent to enter the growing follicle pool. Similarly, in the present study there was evidence of growing follicles in the actively immunized monkey at 24 weeks postimmunization even though the number of primordial follicles had been severely reduced.

Immunological damage to the oocytes left only the follicular clusters within which there is evidence to suggest (Skinner *et al* 1984) that the intercellular junctional complexes and cytoplasmic processes are typical of those found in normal follicular cells (Anderson & Albertini, 1976). It would be interesting to establish the role and fate of the follicular cluster cells remaining after the induction of immunity against the ZP. In all the immunized animals, the ovarian sections revealed the presence of luteinized tissue which was centrally located in the ovaries. It has been suggested that such luteal tissue may represent the spontaneous luteinization of granulosa cells comprising the follicular clusters (Paterson *et al.*1992). Alternatively, the small number of primary, secondary and growing follicles in the ovaries of the immunized

follicles suggests that such luteal tissue might have a follicular origin and represent the spontaneous luteinization of an unruptured follicle or the occurrence of ovulation.

In the present study there was no evidence of macrophage or leucocyte infiltration or inflammation in the ovaries at 2 weeks after booster immunization, which might suggest the absence of cell-mediated immunity. Consistent with this observation, the next chapter (8) cite experimental evidence to suggest that humoral immunity alone can induce ovarian damage. It has been claimed that vaccination with a synthetic mouse zona pellucida peptide $ZP3^{328-342}$ produced long-lasting contraception with a lack of ovarian histopathology. Furthermore, it has been suggested that the lack of ovarian histopathology was due to the absence of zona pellucida T-cell epitopes within that specific ZP3 sequence (Millar *et al.*1989) Additional experiments involving the use of mouse zona pellucida peptides containing T-cell epitope strongly suggested that the primary mechanism of the ovarian histopathology in this species was T-cell mediated (Rhim *et al.*1992). In the later study, a zona pellucida peptide $ZP3^{328-342}$ was found to contain both a T-cell epitope and a B-cell epitope, and induced ovarian histopathology in the immunized animal (Rhim *et al.*1992). Thus, in this murine model both T-cell and humoral responses were potentially important instrument in the induction of ovarian histopathology. Further studies will be needed to determine whether such T-cell mediated response play any role in the ovarian pathology observed in actively immunized primates.

7.5 Summary

Existing data indicated that active immunization against the native ZP3 glycoprotein was associated with the appearance of an ovarian pathology characterized by the accelerated disappearance of primordial follicles. This reduction in the number of

primordial follicles was observed as early as 4 weeks following a booster injection and almost complete depletion of the primordial follicle population was noticed after 32 weeks (224 days) postimmunization. The evidence obtained in this study suggests that the granulosa cells are intact following the destruction of the primordial follicles.

8 Passive Immunization of Marmoset with Anti-Porcine ZP3

8.1 Introduction

The passive administration of anti-ovarian, anti-egg, or anti-zona pellucida antisera to animals has been shown to induce infertility through mechanisms that involve the disruption of sperm-zona interaction during fertilization (Sacco, 1979; Aitken *et al.*1981b). Thus, in the initial studies, antibodies raised against aqueous extracts of ovarian tissue were found to prevent the binding of spermatozoa to the ovum and the antigens responsible for this block were found to reside in the zona pellucida (Sacco & Shivers, 1973a; Garavagno *et al.*1974). This hypothesis was substantiated when mechanically isolated zona pellucida were used to generate antibodies that could block *in vitro* fertilisation in laboratory rodents (Tsunoda & Chang, 1977; Tsunoda & Chang, 1978) by preventing sperm binding to the zona pellucida. Passive immunization with antibodies raised against homologous ovarian antigens in a second species could induce infertility in a variety of animal models such as the hamster (Oikawa & Yanagimachi, 1975), mouse (Jivek & Pavlok, 1975), and rat (Tsunoda & Chang, 1976b). For example, the injection of rabbit anti-hamster ovarian antigen antibodies into female hamsters was associated with the deposition of antibodies on the surface of the zonae pellucidae and an inhibition of fertility that spontaneously reversed after approximately four oestrous cycles. These results emphasize that short-term exposure of a given host to high titre anti-zona antibodies does not irreversibly damage the ovarian tissue but induces a temporary period of reversible infertility. Dean and East (1986) have demonstrated that passive immunizations in the mouse

with antibodies specific for either ZP2 or ZP3, and active in the suppression of fertilization *in vitro,* will also inhibit fertilization *in vivo.* It appears that these particular antibodies did not preclude sperm binding but rather prevented sperm penetration of the zona pellucida through their capacity to cross-link and, hence, stabilize this structure (Dean & East, 1986). The ability of monoclonal antibodies directed against single epitopes on the ZP to block fertilization *in vivo* has also been confirmed in a number of other studies (Isojima *et al.*1984; Koyama *et al.*1985; East *et al.*1985).

The prolonged periods of infertility observed following the induction of passive immunity against the ZP appear to be due to the ability of these antibodies to bind to the zonae pellucidae surrounding follicular as well as ovulated oocytes. As a result, the ova liberated from the ovary over six or seven successive cycles will have been affected by the anti-zona antibodies. Ova collected from passively immunized animals possess an immunoprecipitate on the outer surface of the zona pellucida and an increased resistance to solublization (Tsunoda & Chang, 1976b; Tsunoda & Chang, 1976a; Tsunoda & Chang, 1978). Such observations suggest that the inhibition of both sperm-egg recognition and sperm penetration of the zona pellucida are involved in the mechanisms by which these antibodies disrupt fertility following passive administration.

One of the major problems encountered in the induction of active immunity against the ZP is the disruption of normal ovarian function that has been observed in dog (Mahi Brown *et al.*1982), rabbits (Skinner *et al.*1984) and primates (Paterson *et al.*1992; Sacco *et al.*1991; Upadhyay *et al.*1989). Thus, cynomolgus monkeys immunized with porcine ZP failed to ovulate and also some animals showed massive loss of large antral follicles (Gulyas et al., 1983). Similarly, after active immunization of female rabbits with porcine ZP glycoprotein, there is an interruption of follicular

development which coincides with (i) the stage at which the ZP first appears as an extracellular matrix between the oocyte and follicular cell (Wood *et al.*1981) and (ii) a loss of the ability to ovulate in response to hCG administration (Sacco *et al.*1986). In this species, there is a noticeable reduction in ovarian size and weight as early as 7 weeks after primary immunization (Skinner *et al.*1984). Histopathological analysis of the ovaries of such animals has revealed an increase in the number of follicles with atretic oocytes and complete disappearance of growing follicles as well as corpora lutea within 30 weeks post-immunization. The number of primordial follicles present in long-term (40-48 weeks) immunized animals was also reduced. This decrease was accompanied by an increase in the number of oocyte-free follicular cell clusters. This whole effect appeared to be due to a humoral immune response rather than a cell-mediated response, since there was no sign of macrophage or leucocytic infiltration or inflammation in the ovaries even at the earliest time points (e.g. 2-21 days) examined after the induction of immunity (Skinner *et al.*1984).

In a further study employing the mouse as an animal model, a synthetic ZP peptide containing B-cell, but not T-cell epitopes, coupled to a carrier protein produced long-lasting contraception without any ovarian histopathology (Millar *et al.*1989). A later experiment indicated that a synthetic peptide derived from the mouse ZP3 sequence comprising a 15-amino acid peptide, ZP3 328-342, could elicit a form of ovarian pathology, oophoritis, in B6AF1, BALBc/By, and A/J mice. This synthetic peptide contained both B-cell and T-cell epitopes, suggesting that both epitopes may be involved in autoimmune oophoritis in mice. Moreover, adoptive transfer of the ovarian pathology by lymph node cells and two CD4 T^+ cell lines derived from mice immunized with peptides ZP3 330-342 and ZP3 328-340 strongly suggested that T-cells were involved in induction of oophoritis. While such results clearly implicated a cell-mediated immune reaction is this mouse model, it should be emphasized that the

lymphocytic infiltration that characterizes the oophoritis observed under such conditions, has not been observed in the other species in which ovarian pathology has been induced by active immunization against ZP antigens.

Thus, while the murine studies suggest that the lack of ovarian histopathology or cellular cytotoxicity observed in animals actively immunized with ZP3 peptides may reflect the absence of zona pellucida T-cell epitopes in such vaccines, this generalization may not hold true for other species, including primates (Millar *et al.*1989). The present study was therefore carried out to define the relative importance of B-cell and T-cell ZP3 epitopes in ovarian pathogenicity. Instead of the complicated analysis of different polypeptide fragments of ZP3, adopted by Miller et al (1989) we have addressed this question by determining whether the ovarian pathology recorded in marmosets actively immunized against porcine ZP3 can be transmitted to recipients by the passive transfer of antibody. If this is the case, then it would strongly suggest that humoral immune responses generated through B-cell epitopes are responsible for the induction of ovarian pathology in this primate model, rather than the T-cell epitopes implicated in the murine studies.

8.2 Material and Methods

8.2.1 Passive Immunization

Four sets of sexually mature female marmoset twins from the Medical Research Council, Edinburgh, Primate Colony were used for this study. The experimental protocol made use of the fact that marmosets generally produce twins that are immunologically tolerant of each other owing to antigen exchange that occurs due to the exchange of cells through the placental circulation (Wislocki, 1939). One member from each of 3 sets of twins was actively immunized with porcine ZP3 while the other

member served as a recipient (Active immunization procedure for generation of antisera for this study was same as in Chapter 7.2.2). 2.5ml of antisera, possessing an antibody titre of >1/10,000, from the actively immunized member of each set of twins was transferred to its partner on day 0 and the same amount was also transferred on day 1. In this way, 3 animals received the passive transfer of anti-porcine ZP3 antibodies from an actively immunized co-twin while the 4th set of twins served as the controls, each animal receiving a reciprocal transfer of serum from its partner using exactly the same protocol as was employed for the treated animals.

8.2.2 Histology

One ovary from each animal was removed by laparotomy and unilateral oophorectomy and the second removed at autopsy 4 weeks later. The timing of the initial hemicastration was staggered to occur at weekly intervals. Thus, if the passive administration of the antisera/control sera was given as T=0, ovaries were removed from the first animal on weeks 1 and 4, from the second animal on weeks 2 and 5, and from the third on weeks 3 and 6. In the two control marmoset monkeys, one ovary was removed from each animal one week before the induction of passive immunity while the remaining ovaries were removed at autopsy on weeks 3 and 6, respectively. The purpose of using such a staggered approach was to obtain as much information on the rate at which the ovarian pathology occurred as possible.

All the ovaries were fixed in 4% paraformaldehyde in buffered saline, embedded in paraffin and 5 μm sections were obtained. Every 20th section was stained with haematoxylin and eosin and the primordial follicles were counted using the light microscope. For each ovary the total number of sections obtained varied from 800-1000 and around 50 sections (every 20th) were counted from each ovary.

Primordial follicles were identified on the basis of the presence of an oocyte surrounded by squamous pre-follicular cells.

8.2.3 ELISA Assay

Blood samples were taken at weekly intervals to monitor antibody titres using an enzyme linked immunosorbent assay. The assay was performed as described in section 7.2.2.

8.3 Results

In this study the role of humoral immune responses in mediating the ovarian pathology observed in response to the induction of immunity against porcine ZP3 was investigated using a simple passive immunization approach. As indicated in the Introduction, twins were selected for this study in order to avoid any possible anaphylactic shock due to the large volume of serum administered to each animal and the risk of complications due to the presence of immunological differences between the host and the recipient. The consequences of administering 5.0 ml of a very high titre anti-porcine ZP3 antibody on primordial follicles integrity is illustrated in Figure 8.1.

Within one week of the passive transfer of anti-ZP3 antibodies to the marmoset monkey, the ovarian histology appeared normal exhibiting a large number of primordial follicles in the ovarian cortex and an actively growing follicle population including primary, tertiary and Graafian follicles (Figure 8.2). In all respects the ovary removed from the passively immunized animal at this time appeared to be the same as the control animal and contained very similar numbers of primordial follicles (7617 vs. 7948 for the immunized and control animals respectively.; Figure 8.1 and Figure 8.7).

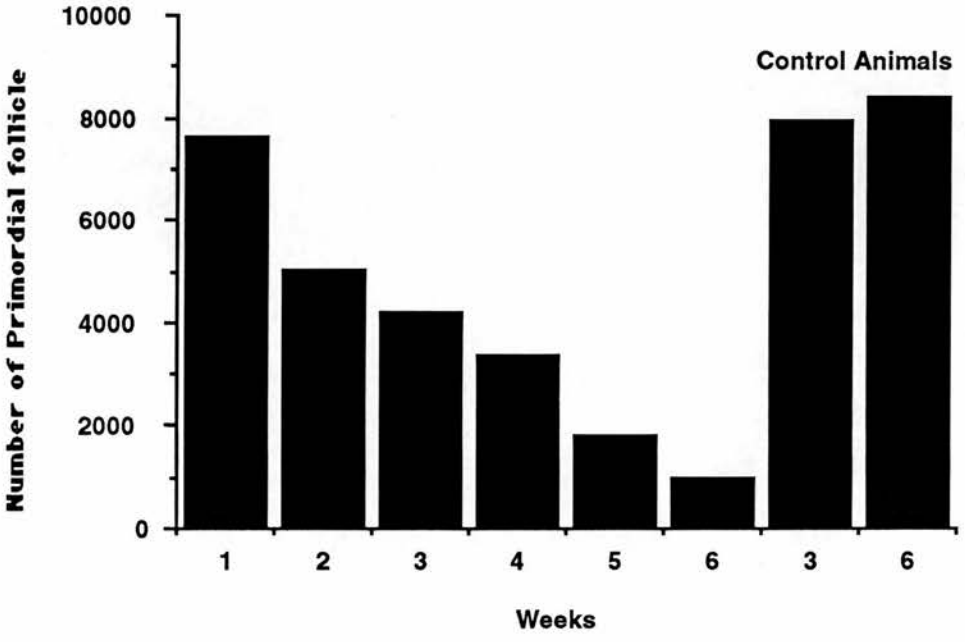


Figure 8.1 Effect of passive immunization on the primordial follicle population of marmoset ovaries at different times.

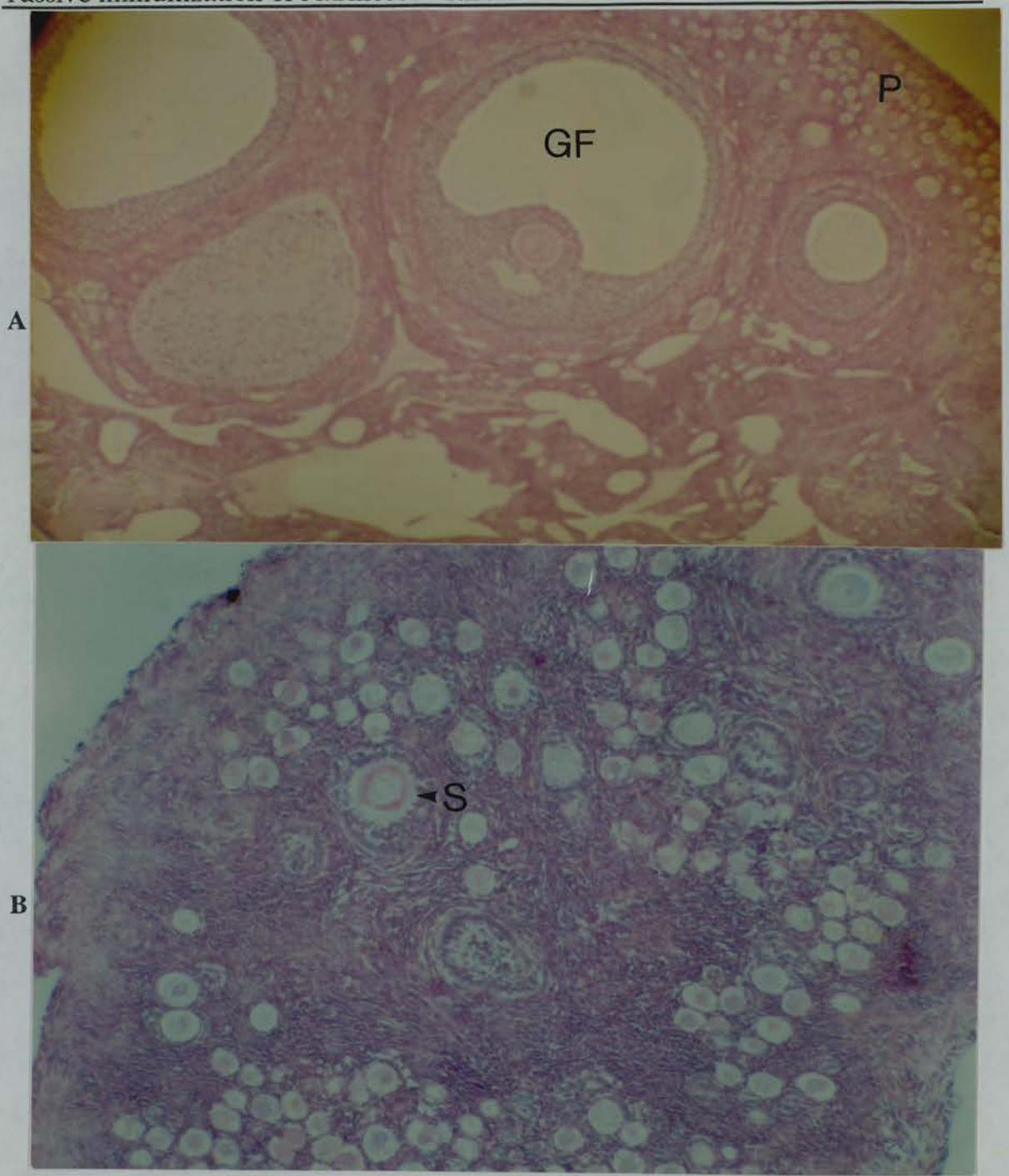


Figure 8.2 Histology section of marmoset ovary after passive transfer (one week) of porcine ZP3 antibody. Marmoset ovarian section stained with haematoxylin and eosin. The ovarian histology appeared normal exhibiting large number of primordial follicles (P) in the ovarian cortex and evidence of primary , secondary (S) and Graafian follicle (GF). (B, 25 x) High magnification of (A, 10 x).

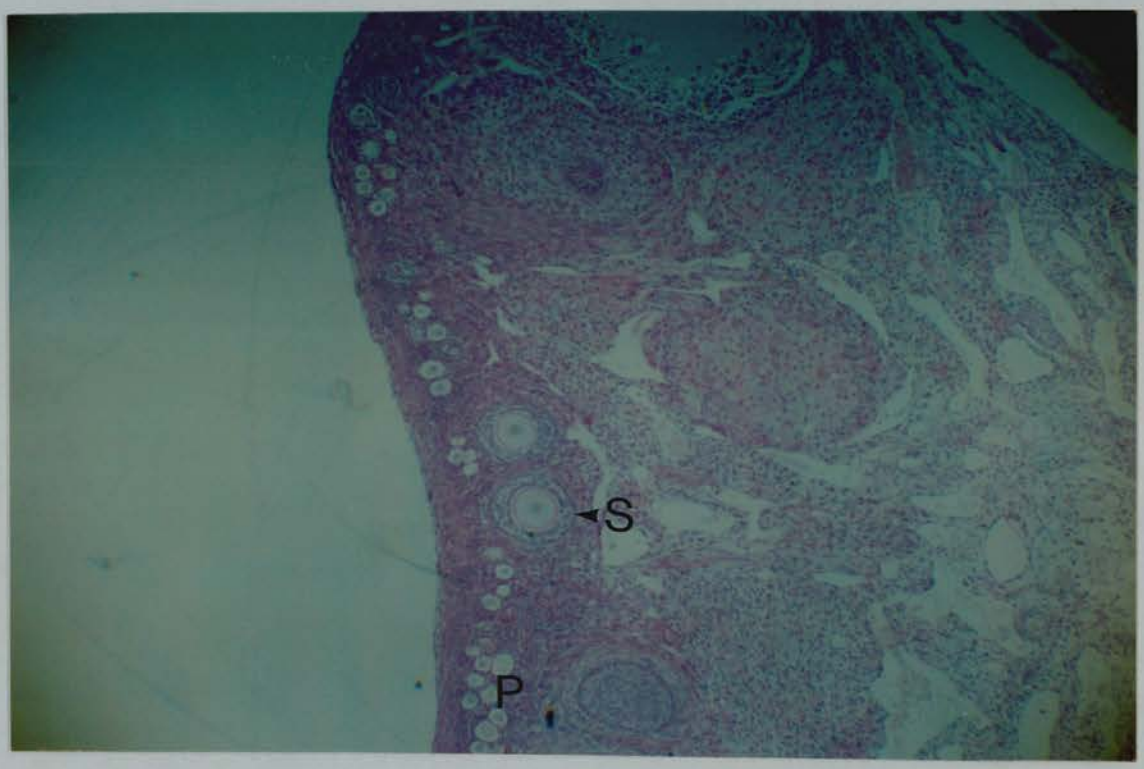


Figure 8.3 Marmoset ovarian section stained with haematoxylin and eosin. Two weeks after the passive transfer of anti-ZP3 antibodies the primordial follicle (P) number had started to decline and there were secondary follicles (S) also present. (10 x)

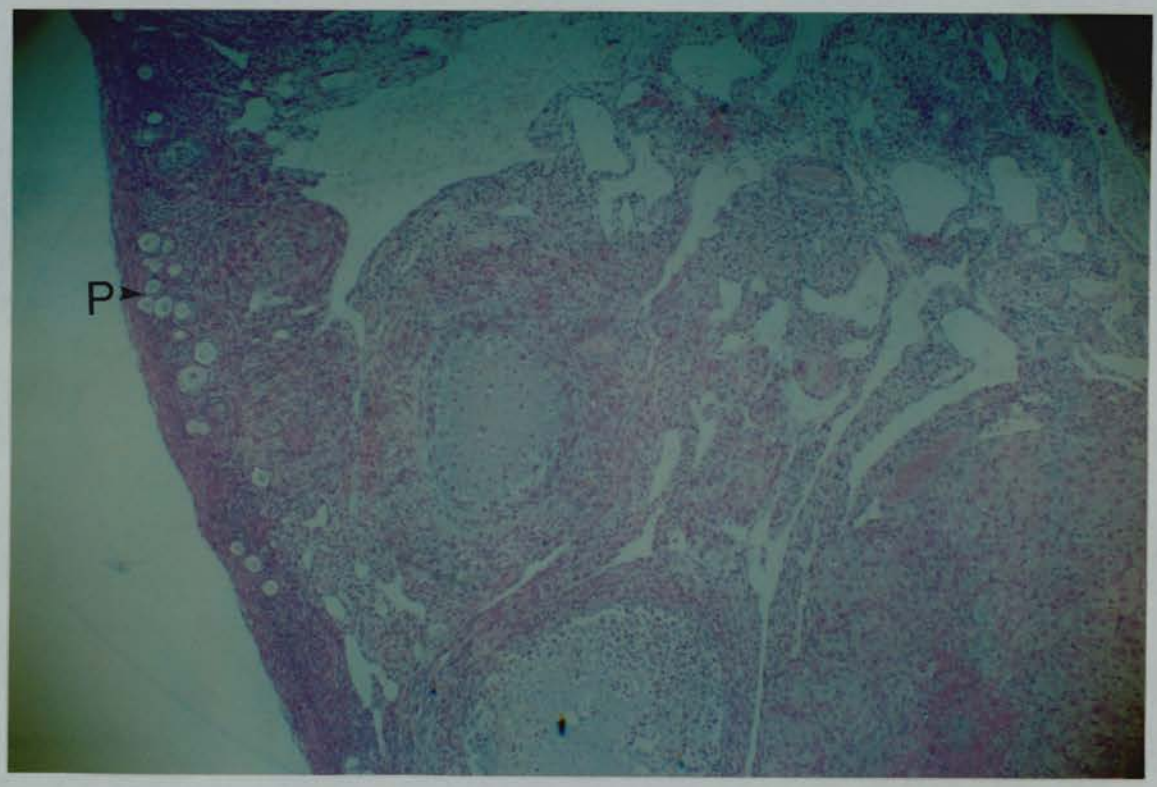


Figure 8.4 Marmoset ovarian section stained with haematoxylin and eosin. Three weeks after the passive transfer of anti-ZP3 antibodies number of primordial follicles (P) observed had declined further. (10 x)

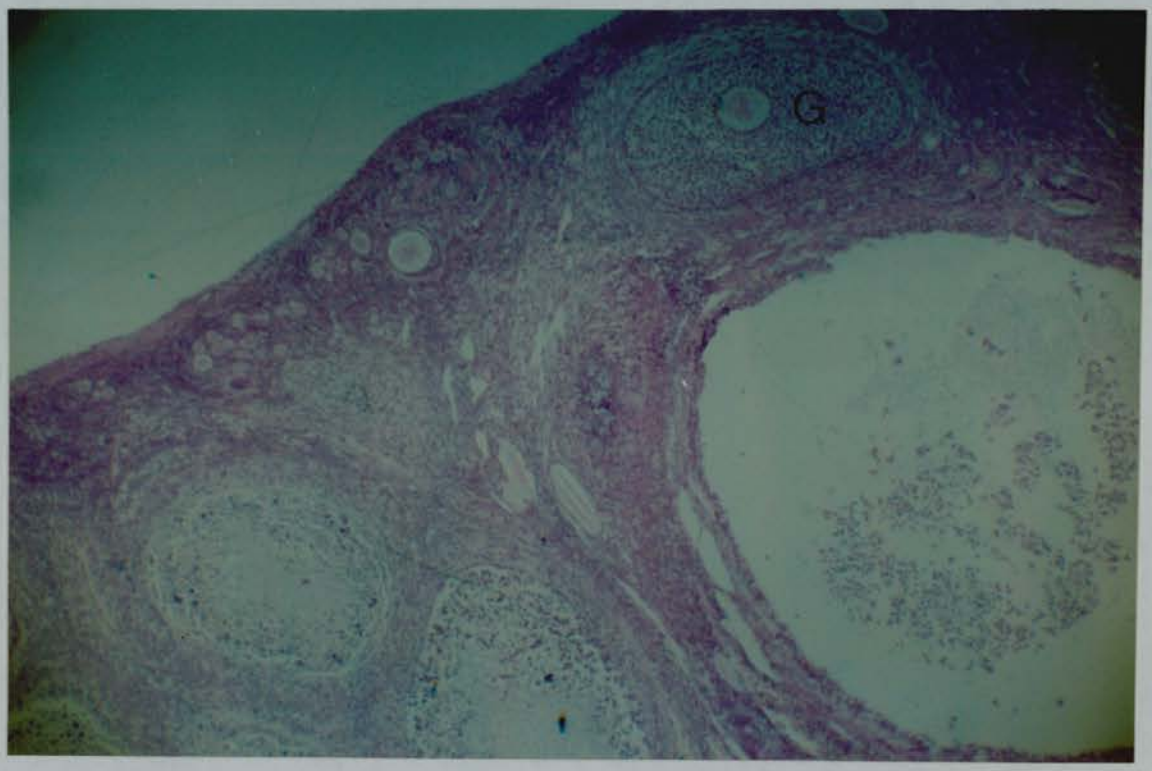


Figure 8.5 Marmoset ovarian section stained with haematoxylin and eosin. Five weeks after the passive transfer of anti-ZP3 antibodies the number of primordial follicles observed had declined and growing follicle was present (G). (10 x)

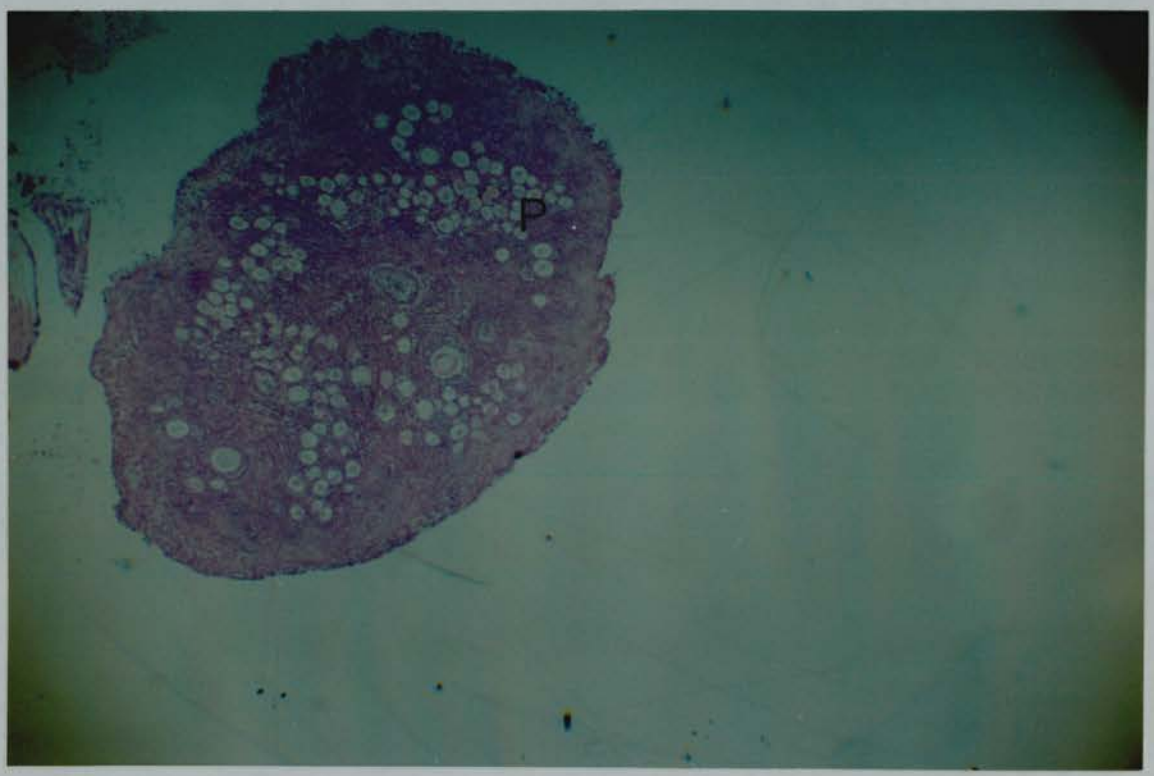


Figure 8.6 Marmoset ovarian section stained with haematoxylin and eosin. Control animal after 6 weeks of study appeared normal exhibiting large number of primordial follicles (P) in the ovary. (10 x)

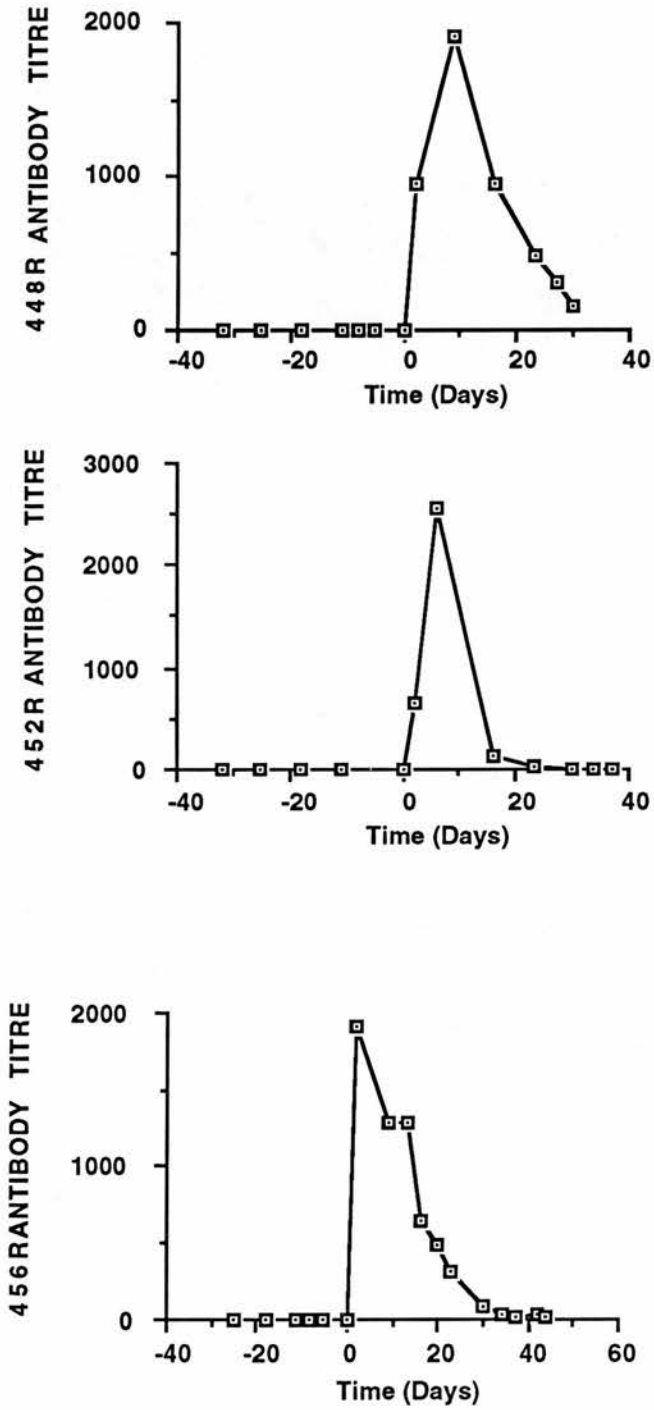


Figure 8.7 Antibody titre of passively immunized animal, analysed by ELISA.

Two weeks after the passive transfer of anti-ZP3 antibodies the primordial follicle number (5020) had started to decline but still there was an actively growing follicle population (Figure 8.3).

Thereafter the number of primordial follicles decreased progressively with time (Figure 8.4) such numbers recorded three, four, five and six weeks after the induction of passive immunity were 4190, 3370, 1839 and 1009 respectively. There was no evidence for the infiltration of mononuclear cells of lymphoid origin into the passively immunized animals at any stage. Five weeks after the passive transfer of anti-ZP3 antibody the number of primordial follicles observed had declined although a population of actively growing follicles was also present (Figure 8.5).

The antibody titres for the passively immunized animals were monitored by an enzyme linked immunosorbent assay. After the transfer of the anti-ZP3 antibodies to the marmosets, the antibody titre detectable in the recipients was in the range 1/1920-1/2560 (Figure 8.7). The antibody titres subsequently started to wane within three weeks of antibody transfer.

8.4 Discussion

It has been demonstrated in a number of animal models that ZP3 is highly immunogenic and able to disrupt fertility either by inhibiting the fertilization process directly or by interfering with the viability and development of the primordial follicle population (Mahi Brown *et al.*1988; Bamezai *et al.*1988; Sacco, 1979; Sacco *et al.*1983; Aitken & Richardson, 1980; Paterson & Aitken, 1989). Many of these studies, particularly those employing porcine ZP antigens, have resulted in the induction of ovarian pathology in a variety of animal models including rabbits, dogs,

squirrel monkeys, bonnet monkeys and marmosets (Skinner *et al.*1984; Mahi Brown *et al.*1988; Sacco *et al.*1991; Paterson *et al.*1992).

In such cases, histopathological examination of the ovaries revealed decreased numbers of primordial follicles, as well as developing follicles and corpora lutea (Mahi Brown *et al.*1985; Sacco *et al.*1987; Skinner *et al.*1984; Paterson *et al.*1992; Upadhyay *et al.*1989). Obviously, either humoral or cell-mediated immune responses to ZP antigens could theoretically have damaging effects on the ovary (Shivers *et al.*1981; Mahi Brown *et al.*1992; Rhim *et al.*1992). In this study, the role of humoral immune responses in the induction of ovarian pathology has been examined. Porcine ZP3 antibodies from marmoset monkeys transferred to a co-twin did not produce any untoward side effects on the normal health of the animals. Moreover, examination of the ovaries for infiltrates of inflammatory cells did not yield any positive finding. However, scrutiny of the ovaries revealed a dramatic loss of primordial follicles which occurred in a relatively short period of time.

Active immunization with glycosylated, wild-type glycoproteins has previously been found to produce profound effects on the ovarian histology of immunized animals such as rabbits (Skinner *et al.*1984), bitches (Mahi Brown *et al.*1988), squirrel monkeys (Sacco *et al.* 1991; Keenan *et al.*1991) and bonnet monkeys (Upadhyay *et al.*1989). These data indicated that glycosylated ZP proteins were highly immunogenic and produced severe disruptions in normal ovarian function. Keenan et al (1991) recently reported that ovarian malfunction in rabbits is dependent on the immunological response to the ZP3ß component of porcine ZP3 and that carbohydrate determinants were probably of critical importance in the pathophysiology of ovarian damage (Millar *et al.*1989). However, Paterson *et al* (1992) have used the deglycosylated peptide core of porcine ZP3ß in marmoset monkeys and have generated antibody titres similar in magnitude to those recorded

with glycosylated ZP3, and yet still recorded a profound effect on ovarian pathology. In contrast, synthetic peptides representing portions of the murine ZP3 molecule which is homologous to porcine ZP3ß, have been reported to be immunogenic in mice when conjugated to a macromolecule carrier and have generated antibodies that had anti-fertility effects without any ovarian pathology (Millar *et al.*, 1989). Furthermore, it has been suggested that the lack of ovarian histopathology was due to the absence of zona pellucida T-cell epitopes within that specific ZP3 sequence (Millar *et al.*1989) Additional experiments involving the use of mouse zona pellucida peptides containing T-cell epitopes strongly suggested that the primary mechanism of the ovarian histopathology in this species was T-cell mediated (Rhim *et al.*1992). In the latter study, a zona pellucida peptide $ZP3^{328-342}$ was found to contain both a T-cell epitope and a B-cell epitope, and induced ovarian histopathology in the immunized animals (Rhim *et al.*1992). Thus, both T-cell and humoral responses were potentially important mediators of ovarian histopathology. Nevertheless, this study has never ruled out that antibody alone could cause ovarian pathology.

The present experiment suggests that the presence of anti-zona antibodies *per se* was sufficient to induce ovarian histopathology, as evidenced by a loss of primordial follicles. The cellular mechanisms underlying the ovarian pathology observed in the marmoset did not appear to involve the same T-cell mediated response that characterizes oophoritis in the mouse, and was not associated with the same lymphocytic infiltration. To this extent, the ovarian pathology observed in the passively immunized marmosets reflects that observed in other species actively immunized with porcine zona antigen, such as the rabbit or squirrel monkey, where the loss of ovarian function is not associated with any overt leucocytic infiltration.

It is, of course, possible that any leucocytic infiltration was extremely short-lived and was not resolved with the frequency of sampling employed in this study. For example,

a short term inflammatory reaction within the ovary might have been sufficient to create an acute phase of oxidative stress during which the primordial follicles became damaged. The fact that X-irradiation of the ovary causes the selective demise of the primordial follicle population (Lacassagne *et al.*1962) suggests that the resting oocyte is particularly susceptive to oxidative stress, since such treatment affects biological tissues through the creation of hydroxyl radicals due to the heterolytic fission of water. Apparently the somatic components of the primordial follicles are much more resistant to the radicals generated on irradiation, since they persist in the ovaries as small islands of follicular cells that appear identical to the follicular clusters that accumulate in the ovary following the induction of immunity against the zona pellucida. Clearly further, more detailed studies will be required in order to resolve whether a transient leucocytic infiltration is in fact a feature of the ovarian pathology observed in the passively immunized marmoset.

A final possibility is that the antibodies inflict a direct attack on the primordial follicles because these cells are competent to express zona pellucida antigen. Clearly the primordial follicle does not excrete zona pellucida antigen in a major way since, by definition, the oocytes in a primordial follicle is not surrounded by a zona pellucida. However, whether the oocyte in such follicles exhibit a low level of ZP antigen expression in a possibility that cannot be excluded. Certainly, in the rabbit there is now good evidence that the 55 kD ZP antigen is secreted by both the follicular cells and the oocyte and that expression is observed in the primordial follicles (Lee & Dunbar, 1993). However studies of ZP3 mRNA transcription and translation in the mouse and marmoset clearly indicate that these activities are not detectable until an oocyte has been recruited from the primordial follicle population. Preliminary analysis of ZP3 antigen expression in the marmoset monkey (Thillai Koothan, unpublished observation) do indicate the presence of traces of immunoreactive

material in follicles that are apparently at the primordial follicle stage, judging from the flattened appearance of the prefollicular cells. Clearly, further, more detailed illustrated studies will be required to resolve this important point.

8.5 Summary

Examination of the ovaries of marmosets passively immunized against porcine ZP3ß revealed a dramatic loss of primordial follicles within a relatively short period of time. This experiment suggests that the presence of anti-zona antibodies *per se* is sufficient to induce ovarian histopathology, as evidenced by the loss of primordial follicles. The cellular mechanisms underlying the ovarian pathology observed in the marmoset did not appear to involve the T-cell. Moreover, the fact that passive transfer of porcine ZP3 antibodies to marmoset monkeys was sufficient to eliminate large numbers of primordial follicles suggests that antibody-dependent cell-mediated cytotoxicity (ADCC) might be involved in this primate model.

9 General Discussion

This thesis focuses on the marmoset homologue of the human sperm receptor protein that has been cloned, sequenced and expressed by means of a bacterial expression system and used to immunize rabbits in an endeavour to initiate an analysis of immunogenicity and antigenicity of recombinant marmoset ZP3. The second part of the thesis deals with the ovarian pathology observed after active and passive immunization with porcine ZP3 and its antibody, respectively.

The zona pellucida is an acellular glycoprotein shell that covers the oocyte and provides functionality in terms of sperm binding, the induction of the acrosome reaction, and the prevention of polyspermy (Wassarman, 1990; Wassarman, 1988b). The zona pellucida is comprised of three to five sulphated glycoproteins, depending on the species, which are secreted to form an extracellular coat around the developing oocyte. The human zona pellucida glycoproteins have apparent molecular weights of Mr=90-110,000, Mr=64-76,000, and Mr=57-73,000 (Shabanowitz & O'Rand, 1988a). The ZP3 molecule serves as a sperm receptor and, because of its important biological function, has been studied extensively as a candidate antigen for contraceptive vaccine development. Heteroimmunization with glycosylated, partially deglycosylated or completely deglycosylated ZP proteins induce antibodies that inhibit or reduce fertility in several species (Millar *et al.*1989; Sacco *et al.*1987; Paterson *et al.*1992). *In vitro* studies with anti-zona antibodies suggest that the prevention of sperm-zona interaction was either through a process of steric hindrance or by direct interaction with the ZP3 domain that binds to the sperm surface (Sacco *et al.*1984; Aitken *et al.*1982) depending on whether the antibodies were raised against homologous or heterologous zona antigens. In addition, anti-zona antibodies can bind to target sites

in the ovary *in vivo* and can interrupt follicular growth at the time the extracellular ZP matrix is synthesized, as well as deplete the primordial follicle population (Skinner *et al.*1984; Paterson *et al.*1992). The precise definition of the nature of the immunological response to the various epitopes presented by the ZP is crucial in defining an approach to contraceptive vaccine development that does not interfere with other aspects of ovarian function (Rhim *et al.*1992).

While the induction of immunity against heterologous zona antigens has generated interesting information, such studies may not accurately reflect the consequences of developing immunity against a homologous peptide. The ideal protocol for the evaluation of a contraceptive vaccine would be one in which an animal model, such as the marmoset monkey, was immunized with homologous ZP3 peptides that exhibited perfect sequence homology with human ZP3. In order to develop such a model the primary amino acid structure of marmoset ZP3 would have to be elucidated and compared with its human counterpart. The present study was designed to achieve this objective.

The introduction of the polymerase chain reaction (PCR) has facilitated many molecular biology procedures like amplification of DNA from a single DNA molecule, rapid sequencing, amplification of small amounts of mRNA by reverse PCR or cDNA amplification, as well as cloning. The purpose of this section of the research was to use such molecular biology techniques to clone and sequence the marmoset ZP3 gene and thence determine the primary amino acid structure of this molecule. In the following sections I shall review the principal findings described in this thesis beginning with the structure and expression of the marmoset ZP3 gene and then discuss the use of this animal model in studies designed to elucidate the behaviour of anti-zona vaccines *in vivo*. Finally, the prospects for generating a contraceptive vaccine that targets the ZP3 molecule will be considered.

Structure of Marmoset ZP3 gene

Mar ZP3 has a single open reading frame 1272 nucleotides in length, which gives rise to a polypeptide core of 424 amino acids, with a calculated molecular mass of 46815. On the basis of the hydrophobicity plot (Kyte & Doolittle, 1982) and amino acid homology, a signal peptide of 22 amino acids could be identified, the four amino acids near the cleavage site sharing the "-3, -1 rule" for predicting signal sequence cleavage sites (Von Heijne, 1986). The primary amino acid structure of marZP3 was found to exhibit a high degree of homology (91%) with the human ZP3 (huZP3) sequence and more than 60% homology with the mouse and hamster sequences. Since it has been demonstrated that the zona pellucida confers relative species specificity to sperm-egg interactions, the high degree of preservation of the encoded human and mouse zona proteins was somewhat unexpected. Nevertheless the existence of this conserved sequence is of great value to the development of a contraceptive vaccine since it permits the use of epitopes in an animal model such as the marmoset that share exact sequence homology with the human.

One consequence of such similarities in the amino acid sequence is that marZP3 also shares with the other species in which this molecule has been characterized a second broad hydrophobic domain near the carboxyl terminus, the function of which is currently unknown. Hydrophobic domains like these are typically present in membrane-spanning regions of proteins. The hydrophobic domains in marmoset ZP3 may play an important role in the intracellular trafficking of these secreted proteins or in their interactions with the extracellular matrix. Similar hydrophobic domains have also been recorded in the carboxyl terminus of other zona proteins in the mouse (ZP2 and ZP3), human (ZP2 and ZP3) hamster (ZP3) and rabbit (Liang *et al.*1990; Ringuette *et al.*1988; Chamberlin & Dean, 1990; Kinloch *et al.*1990; Schwoebel *et al.*1991).

Another highly conserved feature of the ZP3 molecule is the number and location of the cysteine residues; the human, marmoset, mouse and hamster ZP3 sequences all possessing 15 cysteine residues at identical sites. The mouse, human and hamster ZP3 genes contain eight exons each. The coding sequences of the mouse and human ZP3 genes are 74% homologous, and they encode for 424 amino acid peptides that are 67% identical (Chamberlin & Dean, 1990). As indicated overleaf, the primary amino acid structure of marZP3 was found to exhibit a high degree of homology (91%) with the huZP3 sequence and more than 60% homology with the mouse and hamster sequences. The hamster gene encodes a 422 amino acid protein that is 81% identical to mouse ZP3. The 745 amino acid human ZP2 protein is 60% identical to that of its murine counterpart, although the human protein has 745 amino acids compared with 713 amino acids in mouse ZP2. The first 300 bp of the 5' flanking regions of human ZP2 and mouse ZP2 are highly conserved. Furthermore, comparison of the 5' flanking sequences of mouse ZP2, mouse ZP3, human ZP2 and human ZP3 genes have identified five conserved DNA elements. One, element IV, contains a 12 bp domain that is 75% identical among the four genes. These data suggest that a common transcription factor binds to the ZP promoter region and may be involved in the co-ordinated expression of the ZP gene (Liang & Dean, 1993).

Oocyte-specific expression

The expression of the ZP3 gene is restricted to growing oocytes and is not detected in other tissues. Once oocyte growth has commenced, increasing amounts of ZP3 transcripts are detected in the ova of primary and secondary follicles. The amount of ZP3 expression declines dramatically in the fully grown and ovulated eggs, at least in the mouse. It has been observed in ovulated or fertilized ova that the size of the ZP3 mRNA is approximately 200 nt shorter than in growing oocytes. Since the poly(A)

tail length of ZP3 is 200 nt this has led to the notion that the zona mRNAs, like other maternal messages, undergo deadenylation and degradation during meiotic maturation and ovulation.

The first 300 bp upstream of the initiation site of mouse and human ZP2 genes are 70% identical. The TATAA box at position -31 bp and a CCAAT box at position -65 bp in the mouse and human are present at comparable positions. Examination of the upstream regions of human ZP3 indicates that these molecules do not share long stretches of sequence identity but possess five short conserved DNA sequences located at comparable distances from the transcription initiation site. It has been demonstrated that one of these, element IV, is necessary and sufficient for reporter gene expression driven by mouse ZP2 and ZP3 promoters microinjected into oocytes (Millar *et al.*1991).

In contrast, evidence was presented that an oocyte specific, ~60,000 kD protein called OSP-1 binds to the sequence 5'-GATAA-3' within the first 100 bp of mouse ZP3 promoter. The OSP-1 molecule is present only in growing oocytes but not in ovulated eggs or two-cell embryos. Nothing is known about the mechanisms that restrict gene expression to the female germ line. However the fact that the expression of zona genes is restricted to the growth phase of oogenesis suggests that their transcription could be used as a marker for investigating the molecular mechanisms governing gene activation in the oocyte.

Recombinant ZP3 proteins

All previous studies concerning the structure and function of ZP glycoproteins has involved the isolation of native ZP from the ovaries of different animal species. Obtaining ZP material by this procedure was very cumbersome and time consuming. It has not been possible to isolate large quantities of ZP protein from the ovaries of all

mammalian species, particularly the human, for contraceptive vaccine development. The abnormal ovarian cyclicity seen in some ZP immunization studies might also have been due to the presence of impurities in the immunogen (Gulyas *et al.*1983). The approach described in this thesis which involves the production of recombinant proteins in *E.coli* eliminates any possible contaminants from ovarian components in contraceptive vaccine studies.

Marmoset ZP3 has been cloned, sequenced and expressed by means of a bacterial expression system and has been used to immunize rabbits in an endeavour to initiate an analysis of the immunogenicity and antigenicity of the recombinant marmoset ZP3 protein. The bacterially-expressed recombinant marmoset ZP3 fusion protein was 86,000 Da as determined by SDS-PAGE and was soluble in aqueous solution. In contrast, the rabbit recombinant 55 kD ZP protein expressed as a cro-ß-galactosidase bacterial fusion protein formed aggregates and was insoluble in the absence of urea (Schwoebel *et al.*1992). The reasons for this difference could include the different vectors and fusion proteins employed in expressing the protein or it could be that the recombinant 55 kD protein possesses a unique hydrophobic nature and is prone to form aggregates.

Soluble recombinant marmoset ZP3 was purified by single-step DEAE-52 anion exchange chromatography. Earlier attempts to generate antibodies against this purified material were not successful. This might have been anticipated since it had already been established that deglycosylated ZP proteins are less immunogenic than the native or partially deglycosylated structures (Yurewicz *et al.*1987; Keenan *et al.*1991; Bhatnagar *et al.*1992). Moreover, it has been suggested in independent studies that antibodies directed against carbohydrates of the ZP are involved in the immunogenicity of ZP proteins (Sacco *et al.*1986). The fusion protein was therefore conjugated to tetanus toxoid in order to enhance its immunogenicity and in this way

an antibody was successfully generated that recognized the recombinant marmoset ZP3 in the immunoblot.

Native mouse ZP3 obstructs sperm binding to the zona pellucida of ovulated eggs in a dose-dependent manner. This obstruction has been attributed to a class of 3.9-kD O-linked oligosaccharide side chains containing a terminal galactose residue which appears to be critical for sperm binding (Bleil & Wassarman, 1988). After spermatozoa have bound to the ZP, ZP3 induces the sperm acrosome reaction and ZP2 binds to the acrosome-reacted sperm in the secondary phase of sperm-zona interaction (Mortillo & Wassarman, 1991). Embryonal carcinoma cells transfected with the mouse ZP3 gene product inhibited the binding of spermatozoa to ovulated eggs and induced sperm to acrosome-react *in vitro*. On the other hand the hamster ZP3 gene product did not exhibit either sperm receptor or acrosome reaction inducing activity. It was suggested that the embryonal carcinoma cells glycosylate mouse ZP3 and hamster ZP3 genes differently, despite the fact that the primary amino acid structures are very similar (81%) to one another (Kinloch *et al.*1991). In contrast, the data from Beebe et al (1992) have unequivocally demonstrated that the mouse recombinant ZP3 expressed in different cell lines such as rodent or primate cells exhibited the biological activities associated with native ZP3.

Recombinant marmoset ZP3 polypeptide may not exhibit sperm binding and acrosome inducing properties since prokaryotes lack the glycosylation apparatus, but the polypeptide could still be used for contraceptive vaccine development. Some experimental evidence supports the notion that normal ovarian function may be maintained following immunization with certain deglycosylated (ZP3å/DG; ZP3ß/DG) and partially deglycosylated (ZP3å/EBGD) ZP antigens. Antibodies directed against epitopes involving carbohydrate residues on the ZP3 molecule induced major endocrine disturbances, removal of approximately 30-92% of the

carbohydrate (i.e. ZP3å/EBGD; ZP3å/DG; ZP3ß/DG) generated zona antigens that caused no such adverse effects.

Histological observations on the ovaries of rabbits actively immunized with partially deglycosylated ZP3å-EBGD or chemically deglycosylated ZP3å-DG were much more promising since no ovarian pathologies were observed throughout the study (Jones *et al.*1992).

Collectively such data support the investigation of recombinant ZP3 polypeptides as the basis for contraceptive vaccine development since such molecules can be generated in large amounts in a purified form, free of ovarian contaminants. Moreover, any future vaccine is likely to depend on the synthesis of small peptides encoding linear continuous epitopes that are defined by the amino acid sequence. Under such circumstances it is important that non-glycosylated recombinant peptides are used in the vaccine engineering process, even if the antigenicity of such molecules has to be enhanced by conjugation to carrier molecules.

Ovarian Pathology

Antibodies to the ZP have a contraceptive effect by reducing fertility in a number of animal species. Earlier studies suggested that the inhibition of fertility was due to a disruption of sperm binding to the ZP through the ability of anti-zona antibodies to interfere with the sperm receptor function of ZP3. Further studies demonstrated that with some zona antigens the contraceptive effect was associated with the destruction of primordial follicles, failure of ovulation and abnormal reproductive cycles which ultimately rendered the immunized animals sterile (Paterson *et al.*1992). The reasons for the primordial follicle destruction and associated loss of oocytes are not clear since the oocytes contained within primordial follicles do not express ZP antigens. The absence of mononuclear lymphocytes in the ovaries of animals actively and passively

immunized with ZP3 or anti-ZP3 antibodies suggested a lack of cell mediated immune response involvement, in an animal model involving the use of porcine zona antigens to immunize rabbits (Skinner *et al.*1984). Similarly, immunization of mice with rat zonae pellucidae had a significant impact on ovarian follicular development, even though there was no visible evidence of an active inflammatory response (Mahi Brown *et al.*1992). On the other hand, the use of a T-cell proliferation assay indicated an involvement of the cellular compartment of the immune system in dogs actively immunized with porcine zona antigens (Mahi Brown *et al.*1992). In the marmoset, active or passive immunization against porcine ZP3 induced ovarian pathology in the absence of any overt leucocytic response. A similar absence of leucocytic infiltration has been observed in the ovaries of bitches (Mahi Brown *et al.*1988), and rabbits (Skinner *et al.*1984; Jones *et al.*1992) actively immunized with porcine ZP3. However, in all these studies, it is not possible to rule out the possibility that a short lived leucocytic infiltration did occur before the initial observations of the histological normality of the ovaries was undertaken.

The most convincing evidence for a role for cell-mediated immunity in the ovarian pathology observed following the induction of active immunity against ZP3 has come from studies on mice. However the results of such experiments appear to be very strain dependent. Thus, immunization of mice with mouse ZP3 328-343 peptide produced only infertility in NIH mice without any ovarian disease. Interestingly, the same peptide (ZP3 328-343) contains the seven amino acid binding site of an anti-ZP3 monoclonal antibody that causes infertility in female mice (East *et al.*1985). Immunization with mouse ZP3 peptides representing amino acid sequences 183-196, 206-221, 336-351 and 371-398 did not induce oophoritis, whereas the peptide containing mouse ZP3 amino acid sequence 328-343 was a potent inducer of oophoritis in $B6AF_1$ mice. Indication of mild focal infiltration of lymphoid cells

confined to the interstitial spaces began on day 6 of the immunization protocol. On day 10 the disease prevalence was 85%, ovarian follicles were infiltrated by inflammatory cells and the largest antral follicles had inflammatory cells among granulosa cells, adjacent to the ZP and inside the oocytes (Rhim *et al.*1992). The fact that the mouse ZP3 peptide 328-343 could elicit oophoritis in some strains of mice ($B6AF_1$, A/J) but not others, indicated that the susceptibility to this side effect is under genetic control. A unique feature of the peptide is that it contained both a T-cell epitope and a B-cell epitope. Consequently, both T-cell epitope and antibody involvement are implied in the pathogenesis of murine oophoritis. Nevertheless, since the passive transfer of lymph node cells and two $CD4^+$ T-cell lines derived from mice immunized with peptides ZP3 330-342 and ZP3 328-340 induced oophoritis in the T-cell recipients, in the absence of anti-ZP3 antibodies, we must conclude that the primary mechanism responsible for ovarian dysfunction in this model was T-cell mediated. As yet the significance of the B-cell epitope in the mouse ZP3 328-343 peptide in the induction of ovarian pathology has not been determined. Moreover, the fact that passive transfer of porcine ZP3 antibodies to marmoset monkeys was sufficient to eliminate large numbers of primordial follicles suggests that antibody-dependent cell-mediated cytotoxicity (ADCC) might be involved in this primate model. Cells with cytotoxic potential, which posses Fc receptors for IgG, may bind to and lyse target cells coated with antibody of the relevant class. Natural killer cells (NK) might play a role in the so called ADCC model. This possibility could be ruled out by an experiment involving the passive transfer of univalent ZP3 antibodies and then assessing the ovarian status of the immunized animals. Such an experiment is currently being undertaken.

The future of contraceptive development based upon the ZP as a candidate antigen is dependent upon the elimination of the ovarian pathology and associated

irregularities in reproductive cyclicity and hormonal profiles. It seems that anti-ZP antibodies selectively destroy the oocytes in the primordial follicles that secrete the ZP but leave the pre-follicular cells intact. The follicular cells later developed into follicular clusters and could still produce steroid hormones as long as they remained viable in the ovary. Epitope mapping of ZP antigens that are exclusively associated with mature oocytes but not the primordial or developing follicles should facilitate the rational design of contraceptive vaccines that selectively induce antibodies which would attack the mature antral follicles only. Since the zona pellucida significantly increases its binding affinity for spermatozoa in the final stages of oocyte maturation there is every reason to believe that antigenic changes do occur in the zona pellucida in concert with the final stages of oocyte maturation. Such changes in binding affinity are presumably mediated by post-translational alterations to the ZP3 oligosaccharide side chains rather than modifications to the peptide core. In light of such considerations it is possible that in addition to the search for epitopes in the polypeptide backbone of ZP3 a detailed analysis of the carbohydrate structures associated with the zona pellucida could provide new insights into the way in which contraceptive vaccines might be engineered.

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